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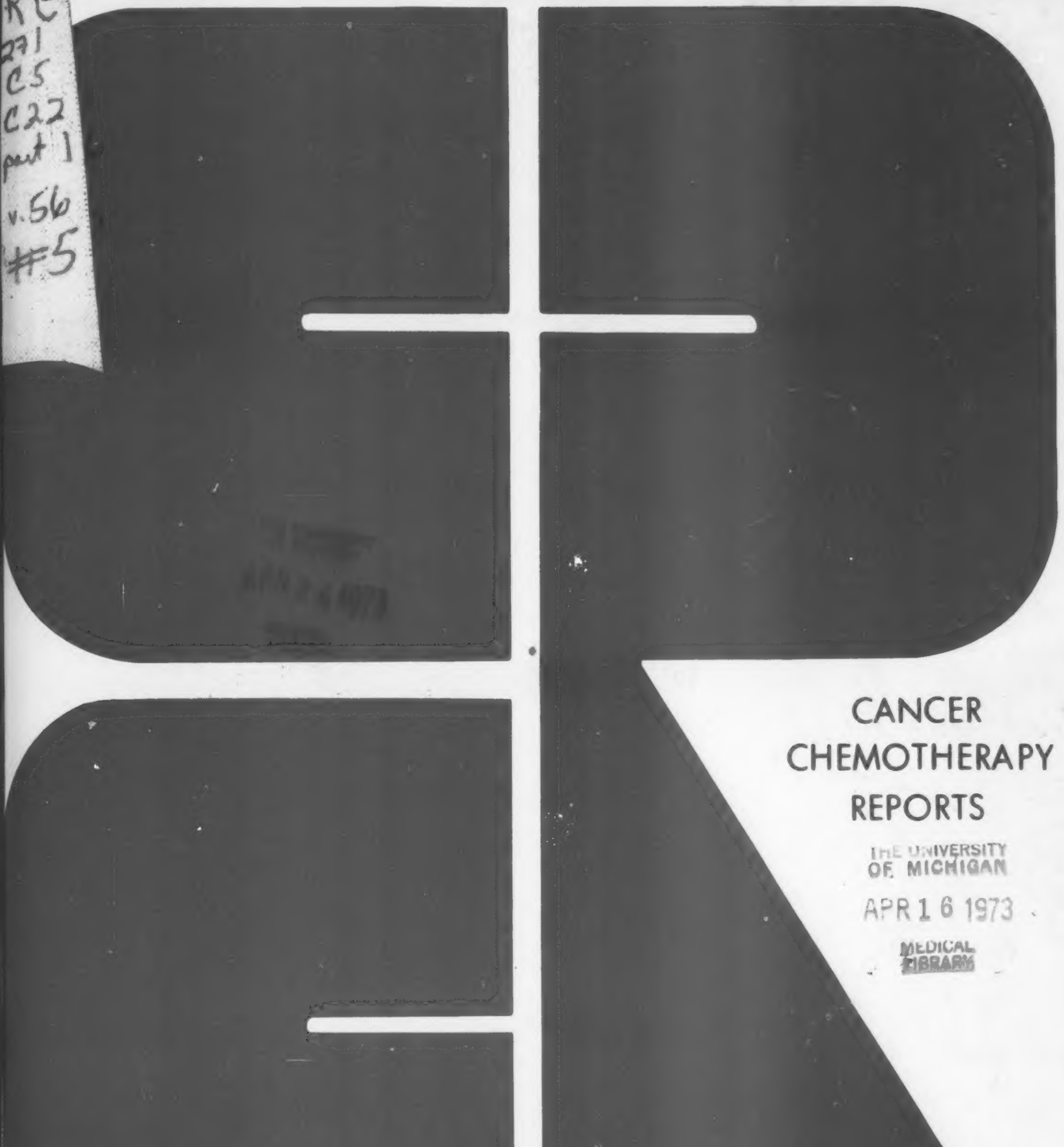

CANCER CHEMOTHERAPY REPORTS

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October 1972
Vol. 56 No. 5

Cancer Chemotherapy Reports

National Cancer Institute

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Cancer Chemotherapy Reports

National Cancer Institute
Frank J. Rauscher, Jr., Director

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LETTER

Inhibition of Cilia Regeneration by Antineoplastic Agents: Delay of Band Migration by Vinblastine (NSC–49842), Griseofulvin (NSC–34533), and β-Peltatin (NSC–24819)[1,2,3]

As reported in *Cancer Chemotherapy Reports* last December (1), we believe that the delay of oral morphogenesis in *Stentor coeruleus,* a common pond water ciliate, can be used to assay the cellular site of activity of new drugs. Inhibitors of DNA synthesis have no effect on synchronized regeneration and drugs interfering with RNA synthesis inhibit only early stages of the regeneration process (2), whereas inhibitors of the mitotic spindle, such as colchicine, delay oral regeneration at all stages. In relation to stage 0, stage 3 is particularly sensitive to vinblastine, demecolcine (Colcemid), and melatonin (3).

Delay in stage 3 by inhibitors of the mitotic spindle can be expressed as an exponential function, $y = kx^n$, where y is the delay in hours and x is the concentration of inhibitor in mols per liter. Most of the compounds tested in this system are negative in the sense that they do not generate specific delay equations at concentrations at which the cells regenerate in synchrony and with no abnormalities. (For further discussion and the list of compounds tested *see* reference 4.) The active compounds whose delay equations have been measured include only demecolcine, podophyllotoxin, and melatonin (5). Although griseofulvin disrupts the mitotic spindle and is additive to demecolcine in the stentor assay (1), to our surprise its activity could not be described by an equation nor was stage 3 more sensitive than stage 0 to low concentrations (5.0–10.9 × 10^{-6} M) of the drug. Of all the podophyllotoxin derivatives tested, only the activity of β-peltatin could be described by an equation.[4]

Stage 3 was more sensitive to given low concentrations of vinblastine than stage 0 (also called protruding anterior frontal field [PAFF], *see* reference 3), and vinblastine, unlike griseofulvin, did delay regeneration reproducibly as a specific function of concentration.

This stage sensitivity can be best illustrated by the following: stage 0 cells in 1 × 10^{-5} M vinblastine behaved as controls; if cells were placed in pond water until they reached stage 3 and were then placed in 1 × 10^{-5} M vinblastine, they were synchronously delayed 12.5–13.0 hours relative to controls. Similarly, cells in stage 0 were synchronously delayed for 16 hours by 2 × 10^{-5} M vinblastine. If cells were permitted to reach stage 3 and were then placed in a 2 × 10^{-5} M concentration of vinblastine, death or asynchronous abnormal regeneration ensued. With neither podophyllotoxin, β-peltatin, nor griseofulvin was this striking stage sensitivity observed.

Thus, as computed by the least squares method, the delay equations for regenerating stentors in stage 3 for these drugs are as follows:

Vinblastine: regeneration delay in hours = 2.2×10^{9} $[\text{vinblastine}]^{1.9}$.

β-Peltatin: regeneration delay in hours = 1.9×10^{23} $[\beta\text{-peltatin}]^{1.8}$.

Griseofulvin: no meaningful equation.

This result, coupled with our observation that melatonin, the animal pineal gland hormone, generated a delay equation (5), has led us to cytologic investigations to determine whether these drugs induce typical "colchicine-arrest" (6). Apparently, the aberrations of the mitotic spindle caused by griseofulvin are far less specific than those induced by colchicine and melatonin.[5] This is consistent with the failure of Wilson (7) to bind griseofulvin to isolated microtubule protein. We believe that these results strengthen the hypothesis that the specific colchicine-induced metaphase-arrest type of disruption of the mitotic spindle can be predicted in compounds that delay oral regeneration in stage 3 stentors as an exponential function of concentration (without inducing abnormality or asynchrony). Such compounds presumably disrupt tubulin assembly into microtubules by binding to microtubule protein (8).

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[1] Chemical nomenclature, NSC numbers, and CAS registry numbers are included in the List of Compounds at the end of the text.

[2] Supported by grant NGR 22–004–025 from NASA.

[3] We are grateful to Professor T. N. Margulis for extensive aid in computation.

[4] Data of LaRue-Honig C; *see* reference 1.

[5] Banerjee S, and Margulis L. Mitotic arrest by melatonin. Submitted for publication, 1972.

LIST OF COMPOUNDS

Colchicine: NSC-757; CAS reg. No. 7006-15-7; benzo-[*a*]heptalen-9(5*H*)-one, 7-acetamido-6,7-dihydro-1,2,-3,10-tetramethoxy-

Demecolcine: NSC-3096; colchicine, *N*-deacetyl-*N*-methyl-; Colcemid

Griseofulvin: NSC-34533; CAS reg. No. 126-07-8; spiro-[benzofuran-2(3*H*),1′-[2]cyclohexene]-3,4′-dione, 7-chloro-2′,4,6-trimethoxy-6′β-methyl-

Melatonin: NSC-56423; CAS reg. No. 73-31-4; acetamide, *N*-[2-(5-methoxyindol-3-yl)ethyl]-

β-Peltatin: NSC-24819; CAS reg. No. 1639-55-0; naphto-[2,3-*d*]-1,3-dioxole-6-carboxylic acid, 5,6,7,8-tetrahydro-9-hydroxy-7-(hydroxymethyl)-5-(3,4,5-trimethoxyphenyl)-, γ-lactone

Podophyllotoxin: NSC-24818; CAS reg. No. 518-28-5; (naphtho[2,3-*d*]-1,3-dioxide-6-carboxylic acid, 5,6,7,8-tetrahydro-8-hydroxy-7-(hydroxymethyl)-5-(3,4,5-trimethoxyphenyl-, γ-lactone)

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

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August 16, 1972

COMMENTARY

Mechanisms of Action of Cancer Chemotherapy Drugs: An Alternative Hypothesis Based on Inhibition of Immunologic Function[1,2,3]

Leonard Sadoff[4,5]

SUMMARY

Although the immunosuppressive effect of cytotoxic agents poses a threat to the host's defense mechanisms, creating a dilemma for the cancer chemotherapist, the immunologic activity of cancer chemotherapy drugs may in all likelihood play an important role in the antineoplastic effect of these agents. The "immunosuppressive" effect of these drugs on the function or formation of blocking antibody may be one of the mechanisms causing tumor regression. The effect of cytotoxic agents on leukopoiesis may explain one of the mechanisms of drug-induced inhibition of the immune response.

[Cancer Chemother Rep 56:559–562, 1972]

The mechanism of action of most cancer chemotherapy drugs is generally thought to involve interference with some phase of cell cycle kinetics resulting in *direct* tumor cell kill. The cell-kill hypothesis was elaborated by Skipper and his colleagues in 1964 and recently restated by DeVita (1,2).

The possibility that other actions of these drugs (known and unknown) might account for their antineoplastic activity has been suggested by Hiatt, Rose, and Weintraub (3–5). To quote Hiatt (3, p 161), "What remains to be answered about all the agents presently in use is not why they do not cure . . . but, rather, why these drugs *work* at all, for . . . the quest for metabolic pathways peculiar to the cancer cell has been unsuccessful."[6]

Rose argued that cancer chemotherapy agents work by producing a temporary immune activating effect or by reversing an immune tolerance toward the tumor. The failure of these agents to work, he postulated, is due to the loss of immune activation or general immune depression. In some cases, this is drug induced. The purpose of this report is to extend Rose's hypothesis in the light of more recent discoveries in the field of cancer immunology.

The phenomenon of immunologic enhancement has recently been brought into sharp focus by the discovery by the Hellströms in 1968 of blocking antibodies in certain tumors in animals and man. They found blocking antibodies in the sera of patients with progressive cancer, but not in the sera of those patients who were in remission (6). Blocking antibodies were found to block the antitumor effect of specifi-

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[1] Received Apr 28, 1972; accepted June 23, 1972.

[2] Supported in part by grant CA-12985 from the National Cancer Institute, National Institutes of Health.

[3] Chemical nomenclature and NSC numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Department of Internal Medicine, Southern California Permanente Medical Group, and Department of Medicine, Kaiser Foundation Hospitals, Los Angeles, Calif.

[5] Dr. Sylvan Cohen (Chief, Pathology Department, Kaiser Foundation Hospital, Panorama City, Calif) photographed and interpreted the microscopic slides.

[6] No italics in original.

cally immune lymphocytes in vitro; thus, blocking antibodies appeared to have a role in the mediation of immunologic enhancement. The Hellströms suggested that inhibition of blocking antibodies might be of therapeutic value in the treatment of cancer.

In 1971, Hickok and his colleagues reported that serum blocking factor in patients with cancer was reduced by leukapheresis (7). The buffy coats were exposed to autologous tumor antigen and then reinfused into the patients. After this procedure serum blocking activity was reduced from 70% to 16%. If Hickok's observations are valid, it is possible to speculate that blocking antibodies are intimately associated with the buffy coats, ie, the white blood cells, of the host.

Recently, Scott and his colleagues suggested that leukopenia per se might be one of the factors that trigger antitumor response. They noted definite inhibition of tumor growth and increases in survival time in rabbits with V-2 carcinoma treated with leukapheresis (compared with control animals) (8). They also quoted several reports in which it was proposed that leukopenia per se is involved in inhibition of tumor growth, prolongation of survival time, and suppression of immunity.

The controversy over whether toxic effects (ie, leukopenia) must occur for therapeutic response to be achieved with cytotoxic agents has not been resolved. Since leukopenia is usually arbitrarily and quantitatively defined, functional or qualitative changes in leukopoiesis caused by cytotoxic agents may be overlooked. If blocking antibodies depend on the integrity of the white blood cells for their function, then inhibition of leukopoiesis by cytotoxic agents may interfere with antibody formation or function or both.

One of the great problems encountered with current cancer chemotherapy is the undesirable immunosuppressive effects of the drugs on the patients. Most, if not all, of the cytotoxic drugs are immunosuppressive to some degree. Yet, in order for tumor regression to occur, immunologic response must increase. *How then does a cancer chemotherapy agent decrease the immune response and increase the immune response at the same time?* Herein lies the best argument for an immunologic basis for cytotoxic drug action.

Recently, Hersh and his colleagues reported that response to drug therapy in patients with acute leukemia could be predicted by immunologic evaluation (9). They found that patients converting from immunoincompetence to immunocompetence during therapy achieved remission. In other words, after treatment with combinations of powerful immunosuppressive agents had been given, the immune status of the responding patients remained intact and in some cases improved.

In addition, we observed that patients with cancer of the breast who responded to treatment with combinations of drugs had increased immunologic response to sensitization with dinitrochlorobenzene (DNCB). Further, histologic examination of regressing tumor revealed lymphocytic infiltration around the tumor edges characteristic of a delayed hypersensitivity reaction (fig 1). We postulated that cytotoxic drugs might inhibit blocking antibodies (produced by bursa-derived [B] cells) (10), thus facilitating a cellular immune response and causing temporary tumor regression. When drug-induced inhibition of thymus-derived (T) cells occurred (10), immune protection would disappear, resulting in the familiar disease progression (fig 2).

The paradoxical effect of drugs used in the treatment of cancer on the immune response appears more rational if the role of blocking antibodies in mechanisms of drug antineoplastic action is considered. Moreover, the temporary nature of therapeutic remissions and the rarity of cures obtained with the current use of these agents is better understood. When blocking antibodies are inhibited, the "killer" lymphocytes of the cellular immune system are unopposed and tumor rejection is stimulated (fig 2). At this point, as noted by Hersh et al, there is an apparent or relative increase in the host's cellular immune responses. When the lymphoid T cells are eventually inhibited, the host is deprived of further immunologic protection and tumor progression occurs. Long term remissions or cures could conceivably occur if the host's cellular immune system could somehow be kept intact.

Admittedly, there is no proof for an immunologic basis for antineoplastic drug action. Some drugs such as L-asparaginase, bleomycin, and streptozotocin are not myelosuppressive and specific drug mechanisms appear to operate. The antineoplastic action of hormones is

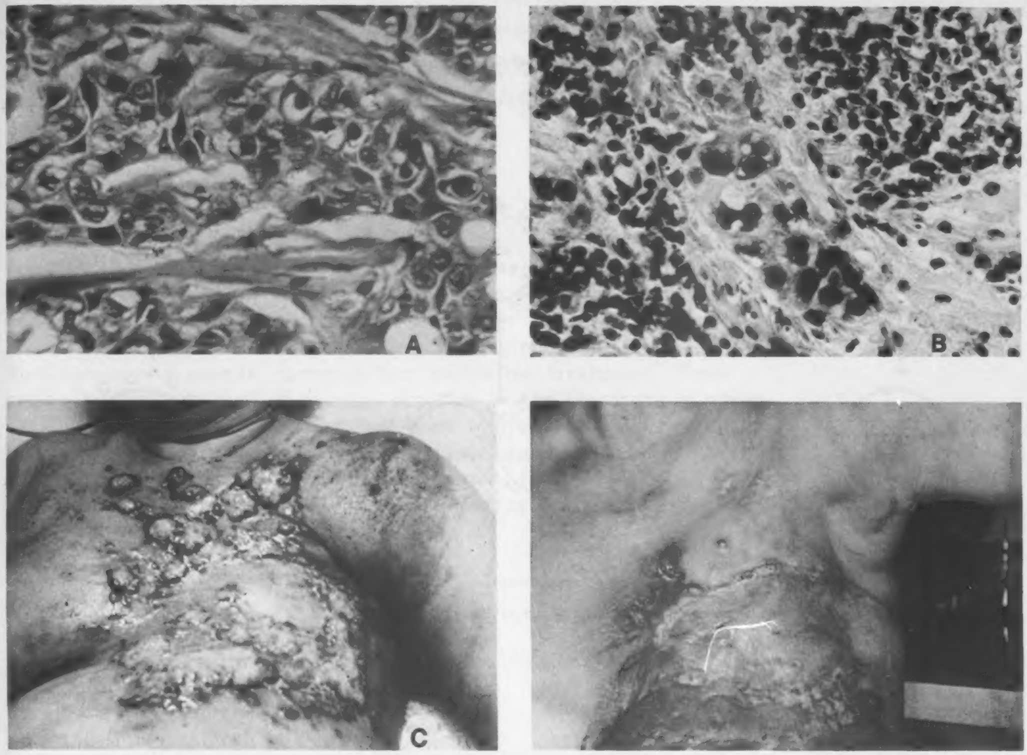

Figure 1.—*A*. Infiltrating ductal carcinoma before treatment (× 450). *B*. Tumor after treatment with cyclophosphamide, vincristine, 5-fluorouracil, and methotrexate. Regressing tumor nodule surrounded by lymphocytic infiltrate (× 450). *C*. Gross appearance of tumor before treatment. *D*. Gross appearance after treatment.

poorly understood. Yet, the other antitumor drugs currently in use have one common action—myelosuppression. Perhaps this action is just an undesirable toxic effect. On the other hand, it might represent the mechanism or one of the mechanisms of antineoplastic effect.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229

Bleomycin: NSC-125066

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; oxazaphosphorine, 2-[bis(2-chloroethyl)amino]-tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Streptozotocin: NSC-85998

Vincristine: NSC-67574; leurocristine, sulfate (1:1); Oncovin

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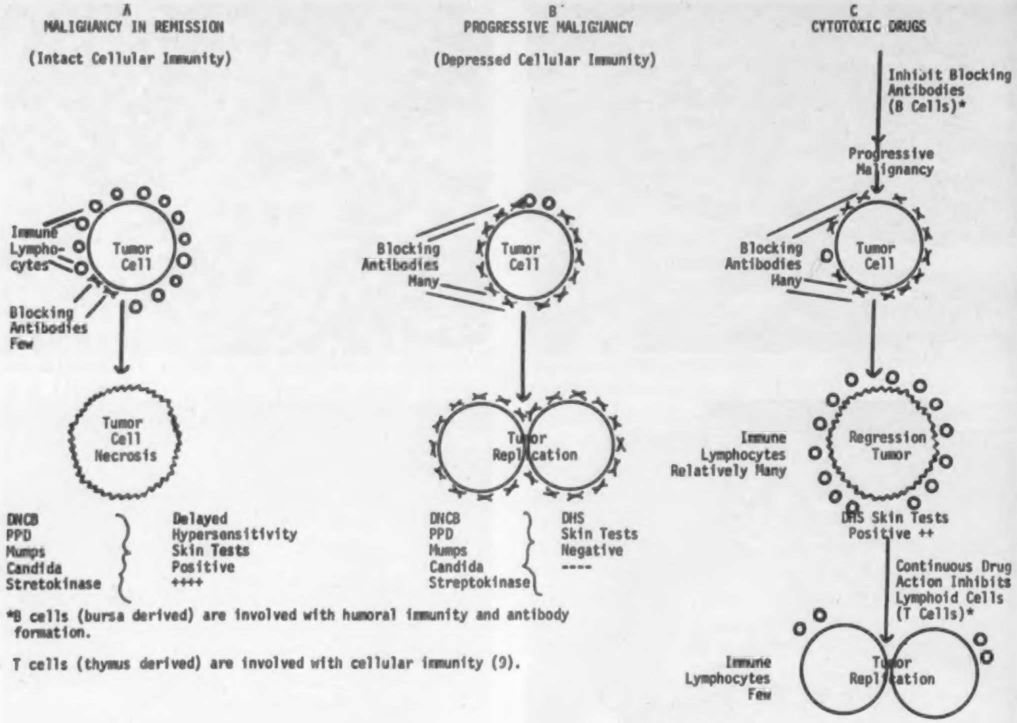
A
MALIGNANCY IN REMISSION
(Intact Cellular Immunity)
Immune Lymphocytes
Tumor Cell
Blocking Antibodies Few
Tumor Cell Necrosis
DNCB
PPD
Mumps
Candida
Stretokinase
Delayed Hypersensitivity Skin Tests Positive ++++
B
PROGRESSIVE MALIGNANCY
(Depressed Cellular Immunity)
Blocking Antibodies Many
Tumor Cell
Tumor Replication
DNCB
PPD
Mumps
Candida
Streptokinase
DHS Skin Tests Negative ----
C
CYTOTOXIC DRUGS
Inhibit Blocking Antibodies (B Cells)*
Progressive Malignancy
Blocking Antibodies Many
Tumor Cell
Immune Lymphocytes Relatively Many
Regression Tumor
DHS Skin Tests Positive ++
Continuous Drug Action Inhibits Lymphoid Cells (T Cells)*
Immune Lymphocytes Few
Tumor Replication

*B cells (bursa derived) are involved with humoral immunity and antibody formation.

T cells (thymus derived) are involved with cellular immunity (9).

FIGURE 2.—Theoretical scheme depicting action of cytotoxic drugs on immune function resulting in tumor regression and later tumor progression.

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In Vivo Determination of the Fractional Kill of Human Tumor Cells by Chemotherapeutic Agents[1,2]

Morris C. Berenbaum[3]

SUMMARY

For a tumor growing at a constant rate and being treated with a regimen of equal doses of a chemotherapeutic agent, the fractional cell kill per dose can be calculated from the growth rates before and during treatment, from the pretreatment growth rate and fractional change in tumor size during the regimen, or from the displacement in growth curves before and after treatment. These methods have been applied to cases reported in the literature in which cyclophosphamide or melphalan was used to treat multiple myeloma and 5-fluorouracil was used to treat rectocolic cancer. The fractional cell kill per dose achieved in these cases varied from 0.14% to 6.5%. It is shown that regimens with such small effects are unlikely to be curative.

[Cancer Chemother Rep 56:563–571, 1972]

If adequate quantitative information were available on the in vivo effects of antitumor agents on human tumor cells and other vulnerable and important cell populations (eg, bone marrow and intestinal epithelium) and on the proliferative kinetics of these various populations, the chemotherapy of cancer could be much more soundly based than it is at present. For laboratory animals, there exists a considerable body of information on the kinetics of normal and malignant cell populations, and quantitative information on the in vivo effects of antitumor agents on these populations is rapidly being accumulated, mainly by more or less direct methods such as counts of tumor cells, measurements of survival times, transfers of tumor cell suspensions to isologous recipients, and measurements of DNA breakdown in vivo (1–6). In spite of the technical and ethical difficulties, there has been a steady accumulation of information on the proliferative kinetics of normal and tumor cell populations in man (7–14). What is lacking is quantitative data on the effects of antitumor agents on these cell populations in vivo, because the direct methods used in laboratory animals are not applicable to man. The purpose of this paper is to show how such data can be obtained for tumor cells in man from the study of patients receiving treatment.

METHODS

There are three basic features of tumor growth and the effects of antitumor drugs which facilitate the sort of analysis undertaken here.

1. The growth rates of many human tumors tend to be constant over long periods (months or years). That is, the fractional increase in size in unit time tends to be constant (15–19).
2. The fraction of a tumor cell population killed by a given dose of a cytotoxic agent tends to be constant (1,20).
3. After destruction by drugs of a fraction of a tumor cell population, the surviving cells often continue to proliferate at the pretreatment rate. This has been shown in tumors

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[2] Supported by grants from the Cancer Research Campaign, the Leukaemia Research Fund, the Nuffield Foundation, and the Medical Research Council.

[3] Wellcome Laboratories of Experimental Pathology, Variety Club Research Wing, St. Mary's Hospital Medical School, London, England.

of both laboratory animals (20,21) and man (22–24).

There are many tumors for which these conditions do not hold, and the effects of departure from these conditions is discussed below. When they do hold, it is easy to show (25,26) that the fate of a population of tumor cells subjected to a regimen of doses equal in size and equally spaced is given by

$$S_n = [S \times 2^{t/T}]^n,$$

where S_n is the fraction of the population surviving n doses and dose intervals, S is the fraction of the population surviving a single dose, t is the interval between doses, and T is the doubling time of the population (fig 1).

Rearranging this equation we have

$$S = \frac{S_n^{1/n}}{2^{t/T}},$$

so that the fractional cell kill (K) per dose is given by

$$K = 1 - \frac{S_n^{1/n}}{2^{t/T}}.$$

This equation allows us to calculate fractional cell kill per dose from net fractional survival after a given number of doses. Further, it is clear from figure 1 that

$$S = [S \times 2^{t/T}]^n = 2^{nt/\gamma}, \tag{1}$$

where γ is the growth rate of the population during the drug regimen. γ may be positive (if the regimen succeeds only in reducing the

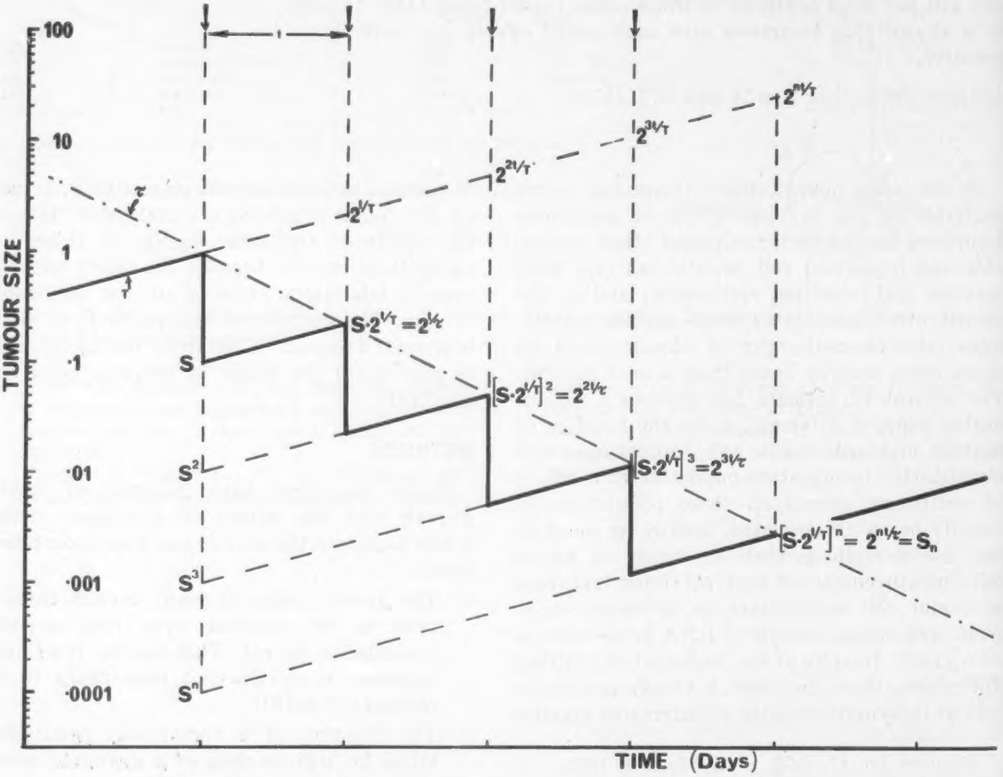

FIGURE 1.—The effect on a tumor with a constant growth rate of a regimen of doses equal in size and equally spaced. Tumor size at the start of therapy is taken as unity. T = doubling time of untreated tumor; γ = doubling time of treated tumor; t = interval between doses; S = fractional survival after a single dose; S_n = fractional survival after n doses. In this example, T = 0.85 days, γ = −0.48 days (ie, the treated tumor has a halving time of 0.48 days), t = 1 day, S = 0.1, and n = 4.

growth rate) or negative (if the regimen converts growth to a decline, as illustrated in figure 1). In the latter case, γ is the halving time of the tumor.

It follows that:

$$K = 1 - 2^{t(1/\gamma - 1/T)}. \qquad (2)$$

This equation allows us to calculate fractional cell kill per dose from tumor doubling time before treatment and its doubling or halving time during treatment. Frequently, however, it is not possible to measure tumor size at the end of treatment or its growth rate during treatment. In such circumstances use is made of the vertical displacement of tumor growth curves before and after treatment.

Figure 1 shows that this displacement gives the value of S^n directly, ie, this is the quotient formed by dividing any value on the posttreatment curve by the vertically corresponding value on the pretreatment curve (either curve may be extrapolated for this purpose). Therefore, provided the doses are equal,

$$K = 1 - \left(\frac{\text{Posttreatment tumor size}}{\text{Projected size without treatment}}\right)^{1/n}. \qquad (3)$$

This equation allows us to calculate fractional cell kill per dose from the vertical displacement of the growth curves and the number of doses. It will be noted that equation (3) includes no expression for dose interval; it was pointed out several years ago by Lajtha et al (27) that, with a tumor growing at a constant rate, the vertical displacement of the growth curves by a series of doses of radiation therapy is independent of the spacing of doses.

In using these methods to calculate fractional cell kill per dose of a tumor cell population in a patient receiving treatment, the appropriate conditions are as follows:

1. The drug regimen during the period over which measurements are made must consist of doses of equal size. The relation between S or extent of kill and dose is not linear for antitumor agents in general, so it is not possible to calculate cell kill per dose for a regimen in which the size of the doses varies during the regimen. (For instance, Breur [22] measured the vertical displacement of tumor growth curves before and after courses of radiation therapy totaling 1000–6000 rads. It appears that the individual doses in a course varied from 70 to 150 rads, so that, although Breur used the data to calculate D37 values for fractionated courses of radiation, it is not possible to use it to calculate cell kill for an individual dose).
2. If equations (1) or (2) are used, the interval between doses must be constant. Equation (3) allows cell kill to be calculated independently of dose intervals.
3. It is desirable to have evidence that the tumor growth rate is constant before treatment and is unchanged afterwards. The latter condition may have to be assumed where tumor growth has not been measured soon after treatment has ended.
4. The measurements of tumor size on which the calculations are based must be sufficiently frequent, must be made over a long enough period, and must be appropriately timed in relation to chemotherapy, so that growth rates and the effects of treatment on them can be assessed with reasonable confidence.
5. Allowance must be made for the fact that, in volume measurements of solid tumors, there may be considerable delay in the removal of dead tumor cells which can largely mask the extent of cell destruction (2,21). Estimates of net survival of tumor cells or growth rates during treatment may therefore be misleadingly high if they are based on measurements of tumor volume made during or soon after short periods of treatment. In such circumstances, it is advisable to use equation (3), the displacement of growth curves before and after treatment being measured once the posttreatment curve has become parallel with the pretreatment curve. This difficulty is not as serious when the tumor population is measured by the serum concentration of a tumor product with a rapid turnover (for instance, a myeloma protein or a hormone), because changes in the number of viable tumor cells can be reflected fairly rapidly by changes in the serum concentration of the product.

RESULTS

These methods will be applied to data taken from papers by Hobbs (24), on the treatment of multiple myeloma with melphalan and cyclophosphamide, and Bru et al (23), on the treatment of rectocolic cancer with 5-fluorouracil.[4] The relevant measurements were made on enlarged photocopies of figures appearing in these papers, and they probably add errors of a few percent to the errors of the original measurements.

Hobbs (24) measured changes in myeloma tumor cell populations by observing the serum concentration of myeloma protein. Data from his figures 6 and 7 are shown here in figures 2 and 3. Calculations of cell kill per dose (table 1) can be made for the first cyclophosphamide regimen (*A*, 150 mg/day for 2 months) and for the last melphalan regimen (*B*, 2 mg/day for 5 months) in figure 2 because measurements of serum myeloma protein were made at the beginning and end of each of these regimens. The other regimens in this figure cannot be used for the purposes of calculation since measurements of serum myeloma protein were not made at the appropriate times. In figure 3 the initial melphalan regimen (*C*, 4 mg on alternate days for 2 months) can be used for calculating cell kill because serum myeloma protein was measured at the start and end of this course. As shown in the figure this tumor recurred after a year or so of therapy, and the doubling time was shorter at the time of recurrence than at the start of treatment. It is uncertain how much the changes in the serum myeloma protein level over the period from the sixth to the 17th month after tumor detection (during which 2 mg of melphalan was given every 2 days, regimen *D*) were due to this emerging clone of tumor cells. However, serum protein levels for the first 3 months of this regimen were probably not unduly affected by this development, and measurements made during this period can therefore be used for the purposes of calculation. The appropriate values for doubling or halving times and net fractional survival times for substitution into equations (1) or (2) are shown in the figures.

Figure 3 from the paper by Bru et al (23) gives measurements of the diameter of a pulmonary deposit of a rectocolic adenocarcinoma. The patient was given three courses of 5-FU (1 g/day for 10 days). Growth was not measured before the first course, and the usable data from the original figure are shown here in

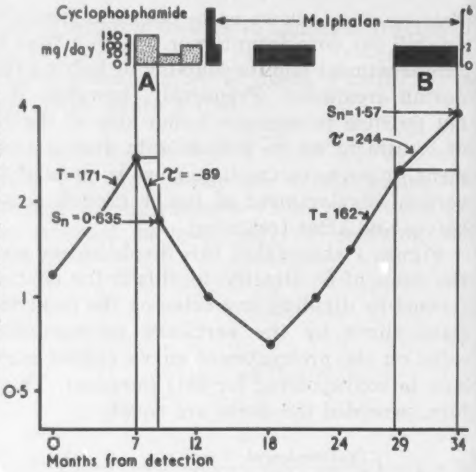

FIGURE 2.—Changes in the size of a myeloma tumor during chemotherapy as measured by changes in serum myeloma protein concentration. Adapted (with permission of publisher) from figure 6 of Hobbs (24). *T* = doubling time in days of untreated tumor; γ = doubling time in days of treated tumor (where tumor size declines during treatment, γ is negative); S_n = net survival at the end of a uniform regimen expressed as a fraction of survival at the start of the regimen.

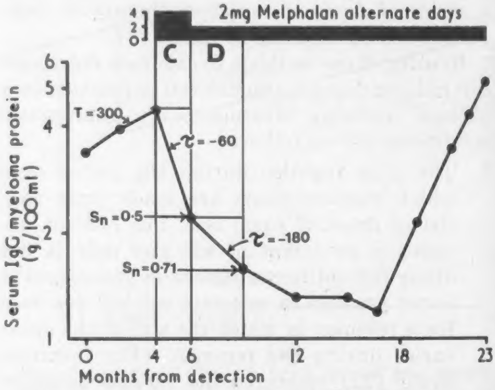

FIGURE 3.—*See* legend for figure 2. Adapted (with permission of publisher) from figure 7 of Hobbs (24).

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[4] Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

TABLE 1.—Calculation of cell kill per dose for human tumors in vivo*

Regimen	Source of data	Drug	Dose (mg)	t (days)	n	T (days)	γ (days)	S_n	S^n	Method of calculation (equation No.)	Percent cell kill per dose
A	Hobbs (24; fig 5)	Cyclophosphamide	150	1	58	171	−89	0.635	—	1 or 2	1.18
B	Hobbs (24; fig 5)	Melphalan	2	1	158	162	+244	1.57	—	1 or 2	0.14
C	Hobbs (24; fig 6)	Melphalan	4	2	31	300	−60	0.50	—	1 or 2	2.7
D	Hobbs (24; fig 6)	Melphalan	2	2	45	300	−180	0.71	—	1 or 2	1.2
E	Bru, et al (23; fig 3)	5-FU	1000	1	10	67	−12	—	0.51	3	6.5
F	Bru, et al (23; fig 3)	5-FU	1000	1	10	72	−13	—	0.54	3	6.0

*Values of n, T, γ, S_n, and S^n (for definitions, *see* text) were measured on enlarged photocopies of figures in the source given. The value of γ in regimens E and F was calculated by substitution of the value for cell kill in equation (2).

figure 4. In view of the brevity of these courses and the delay with which changes in tumor diameter would reflect cell killing, it is not possible to use equations (1) or (2) to measure cell kill. Equation (3) is applicable, however, for the second and third courses (E and F), and the values of S^n are shown in the figure.

The estimates of cell kill per dose based on figures 2–4 are shown in table 1, and vary from 0.14% to 6.5% of the tumor cell population.

DISCUSSION

It is hardly necessary to point out that all three properties of tumors on which these calculations are based (constant growth rate, constant fractional kill, and normal growth of surviving cells) might pertain to only a minority of tumors. Perhaps the weakest part of the argument is the tacit assumption that each of a series of equal doses of an agent applied successively to the same tumor kills an equal fraction of tumor cells. This may not be the usual state of affairs because tumors generally contain a compartment of cells in G_0 that are less sensitive to most agents than cells in cycle (3), and cells in cycle vary in sensitivity at different phases of the cycle (33). For these reasons, drug treatment inevitably changes the relative sizes of the different cell compartments in a tumor population so that the effect of the drug is altered (3,35). Compartmentalization also changes during the natural growth of tumors (34,36), so that estimates of drug effects are valid only for the period during which they are made. It may also be unwise to assume too readily that tumor growth rates remain normal during the intervals between successive doses, especially when the intervals are short.

There are, of course, many tumors with growth rates that are not constant over periods long enough to allow use of these methods. The case of Gompertzian growth curves (28,29) merits further consideration. The growth rates of such tumors fall progressively as the tumors enlarge, and data for Carcinoma 755, Sarcoma 180, and Plasmacytoma 1 (30,31) suggest that, when such tumors are decreased in size by a dose of a chemotherapeutic agent, the remaining fraction of the tumor population tends to adopt the faster growth rate characteristic of the correspondingly smaller tumor, with growth slowing again as the tumor approaches its pretreatment size. One would expect, therefore, that even if equal fractional depletions were produced by successive equal doses of drug, the rate of recovery between doses would increase as the population fell. The eventual fate of the tumor would thus depend on whether, at any particular size, it reached a rate of growth fast enough to compensate for the depletion produced by the last dose. If so, a plateau would be reached and the tumor would tend to remain at this size in spite of continued treatment. If not, the tumor would continue to decrease in size as treatment was continued. In this way, the behavior of such tumors resembles that of

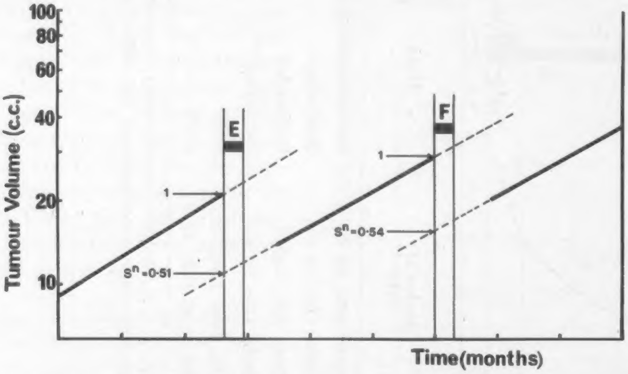

FIGURE 4.—Changes in size of a pulmonary metastasis from a rectocolic cancer during 2 courses of treatment with 5-FU (E and F), 1 g/day × 10. Adapted (with permission of publisher) from figure 3 of Bru et al (23). Values of S^n found by extrapolation of posttreatment growth curves.

homeostatically controlled populations on exposure to repeated doses of drugs (25,26). It is relevant that Salmon (32), in a preliminary communication, reports that the growth of multiple myeloma in man may be described by Gompertzian equations, and that the amount of tumor tends to reach a plateau after several months of continued chemotherapy.

The lifespan of most human tumors, however, is measured in terms of many years, and, although growth may be Gompertzian rather than strictly exponential over the whole span, it matters little for the purpose of calculating cell kill which type of curve is assumed (provided the measurements for any particular calculation are restricted to periods of a few months as they are in the examples described here). Over such periods, which are short in relation to the whole lifespan of the tumor, the growth curves of human tumors are often not detectably different from exponential growth curves (15–19). For the reasons discussed above (drug-induced alterations of drug effect, changes in drug response with tumor growth, and departure from exponential growth), it would clearly be wrong to assume that measurements of fractional cell kill made at a particular time were more generally valid, for instance, for longer periods of therapy or for treatment at other times in the life of the tumor.

The methods presented here, like all such indirect and mathematically based approaches, are indeed beset with uncertainties. Their justification is not that they lead to an accurate and confident estimate of fractional cell kill, but that there is at present no more direct method applicable to man, and that, without even crude estimates of this nature, it is difficult to evaluate the effects of chemotherapy on human tumors except in terms of end results.

Perhaps the most striking finding to emerge from this analysis is the relatively small extent of cell killing by the doses of drugs used. In no case was more than a small percentage of the tumor cell population destroyed by a single dose. It would be interesting to calculate what length of therapy would be required to achieve a cure under such circumstances but, as emphasized earlier, it is most unlikely that either cell kill per dose or growth rate between doses will remain constant during therapeutic periods materially longer than those used for the calculations. On the one hand, cell kill may increase as cells in G_0 are recruited into the cycle, and immunologic defenses may become increasingly effective as tumor size is reduced. On the other hand, the increased sensitivity of the tumor due to recruitment of G_0 cells into proliferation may, with some drug regimens, be more than counterbalanced by the population-expanding effect of such recruitment, and the immunosuppressive effects of the regimen may nullify any immunologic advantage of reduced tumor size. For these reasons, the following calculations must be regarded as no more than conjectural, indicating very broadly the possibilities of cure with standard regimens.

If a chemotherapeutic regimen succeeds in causing a tumor of 10^n cells to decrease in size with a volume halving time γ, then the duration (D) of the regimen required to reduce the tumor to vanishing point (1 cell) is given by

$$D = \frac{n\gamma}{\log 2}\ .$$

This equation is plotted in figure 5 for various values of n. To eliminate a tumor of 10 g (about 10^{10} cells) it would have been necessary to use the most effective regimen (1 g of 5-FU daily, which produced a halving time of 12–13 days) over a year of continuous therapy. This regimen, however, was maintained only for 10 days at a time, and successive courses were separated by intervals of 2–3 months, presumably to avoid excessive toxicity. In the other examples, where halving times of 60–180 days were achieved by therapy, it would have taken 3 ½–9 years of continuous treatment to eliminate a 1-mg tumor (about 10^6 cells), during which time drug resistance would probably have occurred.

If our examples of fractional cell kill are representative of those achieved generally by conventional chemotherapy used against solid tumors in man, it is not surprising that the most we can usually hope for is a prolongation of life, not a cure. It would be of interest to use the methods described here to determine fractional cell killing in massive dose regimens.

We are still a long way from the construction of adequate dose-response curves for human tumors, but the stepwise collection of data in the way outlined here might enable us to determine the general forms of the dose-response curves for particular agents used in man. Such information could provide a basis for a more

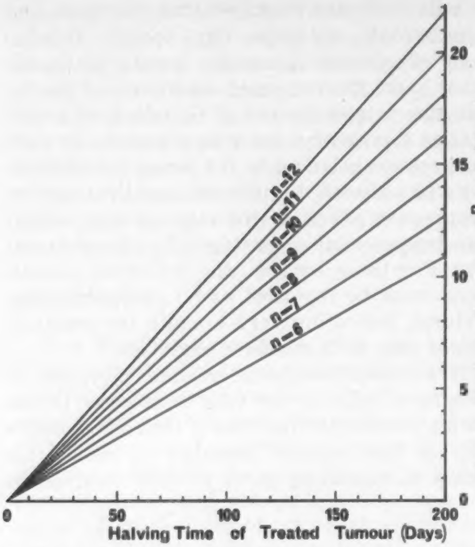

FIGURE 5.—Relation between tumor size, tumor halving time during chemotherapy, and the time required for chemotherapy to eliminate the tumor.

rational planning of chemotherapy for the individual patient than has been possible hitherto.

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Plasma Camptothecin (NSC-100880) Levels During a 5-Day Course of Treatment: Relation to Dose and Toxicity[1,2]

Patrick J. Creaven,[3] Larry M. Allen,[3] and Franco M. Muggia [3,4,5]

SUMMARY

A simplified method of determining plasma camptothecin levels has been developed. Minimum plasma camptothecin levels (C_{min}) during each 24-hour period of a 5-day course of daily treatment were determined in 15 patients to study the relation between C_{min} values and the development of hematologic toxicity. Mean C_{min} values ranged from 0.17 to 1.23 μg of camptothecin/ml of plasma and did not appear to be correlated with dose in the range of doses studied (6.25–20 mg/m^2/day × 5). Correlation of mean C_{min} values with hematologic toxicity was fair for 13 patients in whom toxicity could be evaluated. One of the eight patients in whom toxicity did not occur had a mean C_{min} value of more than 0.5 μg/ml; one of the five patients in whom hematologic toxicity did occur had a C_{min} value of less than 0.5 μg/ml. Although camptothecin is highly bound to human serum albumin (HSA) (equilibrium dialysis indicates that the binding constant is 6×10^7 M^{-1} and that the value for $\bar{n}$ [the number of mols of camptothecin bound per mol of protein] is 0.8), toxicity was not related to HSA concentration, total plasma protein levels, or the use of drugs which bind to HSA. We conclude that daily measurement of C_{min} levels is useful in monitoring daily treatment with camptothecin although it is not an absolute predictor of toxicity.

[Cancer Chemother Rep 56:573–578, 1972]

Camptothecin (fig 1), an antineoplastic agent of novel structure, has been administered in phase I clinical trials in single doses (1), and in weekly and daily (× 5) doses (2). In phase II study, it has been evaluated in the treatment of carcinoma of the colon (3). In initial clinical trials, the toxicity of the drug has been unpredictable, particularly when the drug is given in daily courses of treatment (3). We have, therefore, developed a simplified method for the measurement of camptothecin in the plasma and urine. In this study, we measured plasma camptothecin levels 24 hours after the administration of each dose of the 5-day course to determine whether plasma camptothecin levels could be used as a predictive index of expected or incipient toxicity on which basis the administration of the drug could be monitored and controlled.

MATERIALS AND METHODS

Camptothecin was administered intravenously (iv) in the form supplied by the NCI. It gave a single fluorescent spot on thin-layer chromatography on silica gel G in chloroform-

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[1] Received Mar 3, 1972; revised July 5, 1972.

[2] Camptothecin: CAS reg. No. 25387-67-1. Supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (NCI).

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[5] The expert technical assistance of Mrs. G. Friedman is gratefully acknowledged.

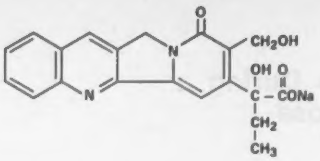

FIGURE 1.—Structure of camptothecin.

ethanol (15:1, v/v) and in ethyl acetate-isopropanol (1:1, v/v).

Patients

The patients in this study were those entered into our phase I evaluation of daily (× 5) camptothecin treatment (2). Since toxicity is known to be increased in the presence of renal disease or extracellular fluid expansion (1), patients with elevated blood urea nitrogen (BUN) values or edema were excluded. Therefore, one patient (No. 5) who became azotemic shortly after the start of treatment received only three of the five doses planned. Five-ml samples of blood were drawn from each patient immediately before the administration of each dose of the drug and 24 hours after the administration of the fifth dose. The blood was drawn into heparinized tubes. After the blood was centrifuged, the plasma was removed, and camptothecin content was estimated by the procedure described in the next section. Hematologic toxicity was defined as a white blood cell count (wbc) of less than 5000 cells/mm^3 or a platelet count of less than 100,000 platelets/mm^3. Since in one instance toxicity was first recorded as late as 2 weeks after the beginning of the course, the drug was considered nontoxic only in those patients with normal counts who were still alive on Day 15.

Estimation of Camptothecin Levels

Samples of plasma (0.5 ml) were placed in 15-ml conical centrifuge tubes (ground-glass stoppers) and 0.5 ml of 3N HCl and 2 ml of water-saturated toluene (analytical grade) were added to each sample. The tubes were shaken for 1 minute and centrifuged at 2000 × *g* for 10 minutes, and the fluorescence of the toluene layer was measured at $\lambda_{\text{Excitation (EXC)}}$ 378 nm, $\lambda_{\text{Fluorescence (FL)}}$425 nm in an Aminco-Bowman spectrophotofluorometer using a slit arrangement to give an 11-nm bandpass on the excitation monochromator and a 5.5-nm bandpass on the emission monochromator. The spectra of the toluene extract of blank plasma and of plasma to which camptothecin (1 μg/ml) had been added are shown in figure 2. Ninety-eight to one hundred percent of the camptothecin was recovered from the plasma. The sensitivity of the method was 10 ng/ml. Blank plasma and plasma to which known amounts of camptothecin had been added were run through the assay procedure with each plasma sample assayed. Standards of camptothecin in plasma and camptothecin in water (1.0 μg/ml) are stable for at least 1 week. Solutions containing 100 μg/ml of camptothecin are not stable, however, and white crystals are precipitated when the solution is left standing.

When acidified plasma from patients who had received camptothecin was treated with 20 ml of toluene and the toluene extract was allowed to evaporate to a small volume, total chromatography on silica gel G in chloroform-ethanol (15:1, v/v) and in ethyl acetate-isopropanol (1:1, v/v) revealed a single fluorescent spot which co-chromatographed with camptothecin.

Binding of Camptothecin to Plasma Proteins

The binding of camptothecin to plasma proteins was investigated by gel filtration, filtration through Centriflo cones, and equilibrium dialysis. For gel filtration, 4-ml samples of plasma containing 2.1 μg of camptothecin/ml were applied to a 2 × 40-cm column of Bio Gel

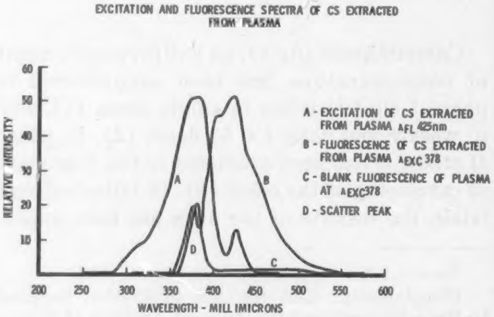

FIGURE 2.—Plasma (0.5 ml) containing camptothecin (*CS*) (1 μg/ml) was extracted with 2 ml of water-saturated toluene and the excitation and fluorescence spectra of the extract were recorded on an Aminco-Bowman spectrophotofluorometer using a slit arrangement to give an 11-nm bandpass on the excitation monochromator and a 5.5-nm bandpass on the emission monochromator.

P-2 (200–400 mesh) (BioRad Laboratories, Richmond, Calif) and eluted with water using an LKB peristaltic pump. The effluent was continuously monitored at 254 nm by a Uvicord II spectrophotometer (LKB Instruments, Inc., Rockville, Md). Fractions (10.5 ml) were collected and monitored manually at λ_{EXC} 373 nm, λ_{FL} 445 nm.

For filtration, 5-ml samples of plasma to which camptothecin had been added in concentrations ranging from 22 to 88 μg/ml were placed in Centriflo cones (Amicon Corporation, Lexington, Mass) and centrifuged at 1000 × *g* for 45 minutes. The camptothecin content of each filtrate was estimated by the method described previously. Loss of camptothecin on the cones was 2.5%.

For equilibrium dialysis, 10-ml samples of 0.4% (0.58 μmol) human serum albumin (HSA), in 0.67 M sodium phosphate buffer (pH 7.4), were dialyzed for 24 hours (with shaking) against 30 ml of camptothecin (2–21 μg/ml) in the same buffer.

The amount of camptothecin bound to HSA was determined spectrofluorometrically by the disappearance of camptothecin from the outer compartment and was corrected for dilution and bag binding.

The data were fitted graphically to a Scatchard plot (5) of $\bar{n}/A$ versus $\bar{n}$ using the equation:

$$\frac{\bar{n}}{A} = kN - k\bar{n},$$

where $\bar{n}$ is the number of mols of camptothecin bound per mol of protein, A is the concentration of unbound drug, k is the intrinsic association constant, and N is the number of binding sites when $\frac{\bar{n}}{A}$ approaches zero. N, the number of binding sites, was determined by the $\bar{n}$-intercept of a line drawn as a tangent to the initial slope of the binding curve. The intrinsic association constant k was determined from the slope of the same tangent line.

RESULTS

The results of gel filtration indicate that camptothecin is highly bound to plasma protein (fig 3). Equilibrium dialysis indicates a binding constant of 6×10^7 M^{-1} and a value for $\bar{n}$ of 0.8, indicating a binding of approximately one mol of camptothecin per mol of HSA. The values for the binding of camptothecin to plasma proteins indicated by studies with Centriflo filter cones are shown in table 1.

TABLE 1.—Binding of camptothecin to plasma proteins*

Concentration of camptothecin (μg/ml)	Percent bound	Percent free
88	99.04	0.96
66	99.27	0.73
44	99.55	0.45
22	99.64	0.36

*Five-ml samples of human plasma containing 22–88 μg of camptothecin/ml were centrifuged at 1000 × *g* in Centriflo cones for 45 minutes. The camptothecin content of each filtrate was then estimated spectrophotometrically.

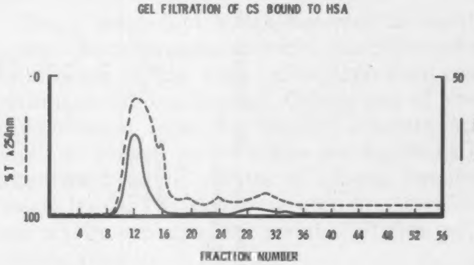

FIGURE 3.—The effluent from a 2 × 40-cm column of Bio Gel P-2 (200–400 mesh) to which plasma containing camptothecin (CS) (2.1 μg/ml) had been applied was monitored continuously at 254 nm (interrupted line) and manually for fluorescence at λ_{FL} 445 nm (λ_{EXC} 378 nm) (continuous line).

The values for plasma camptothecin levels immediately before each dose and 24 hours after the fifth dose of the daily × 5 course of treatment are shown in table 2. Other parameters which could be relevant to the development of toxicity are shown also. Although the numbers at the lower doses are small, there appears to be little relation between plasma camptothecin levels and the dose given. Only one of the four patients who received 15 mg/m²/day had average C_{min} levels higher than the lowest value found in four patients who received 6.5–11.2 mg/m²/day, and this patient had azotemia and did not receive a full course of treatment. Two of seven patients who received 20 mg/m²/day also had average C_{min} levels lower than this value.

TABLE 2.—Dose of camptothecin, plasma C_{min}, and toxicity in patients receiving camptothecin daily × 5*

Patient No.	Dose (mg/m²/day)	Concentration of camptothecin (μg/ml) in plasma after— 24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	Average	Serum albumin (g/100 ml)	Total serum protein (g/100 ml)	Serum creatinine (mg/100 ml)	BUN (mg/100 ml)	Urine volume (average ml/day)	Hematologic toxicity†
1	6.25	0.23	0.38	0.42	0.38	0.31	0.34	3.2	7.1		16	1514	—
2	8.5	0.32	0.33	0.44	0.50	0.69	0.46	—	—		—	403	—
3	11.2	0.30	0.40	0.29	0.46	0.38	0.38	3.6	6.9		17	‡	—
4	11.2	0.61	0.77	0.99	1.19	‡	0.87	3.3	8.4		10	632	—
5	15	0.92	1.52	1.43§		—	1.29	—	—	1.7	70	1272	NE
6	15	0.13	0.19	0.21	0.27	0.23	0.21	2.5	6.6	0.9	17	2601	—
7	15	0.1	0.27	0.35	0.52	0.45	0.34	4.0	8.3	1.0	10	‡	—
8	15	0.22	0.33	0.44	0.53	‡	0.38	—	—	1.3	13	580	NE
9	20	0.23	0.27	0.28	0.36	0.43	0.31	3.1	7.8	0.6	15	300	+
10	20	0.53	0.72	0.55	0.39	0.54	0.55	3.3	7.4	0.7	15	454	+
11	20	0.1	0.45	0.74	0.46	0.67	0.48	2.9	6.6	0.7	11	1604	−
12	20	0.06	0.17	0.26	0.16	0.18	0.17	3.7	—	0.7	13	1582	−
13	20	0.78	0.88	1.0	1.14	**	0.95	3.4	7.8	1.0	11	380	+
14	20	1.15	1.34	1.32	1.1	1.24	1.23	3.1	7.8	0.8	11	1140	+
15	20††	0.28	0.82	1.40	0.80	0.76	0.81	2.1	5.8	0.6	9	‡	+

*Camptothecin was administered iv daily × 5 as a single injection. Immediately before the injection, 5 ml of blood was drawn and placed in a heparinized tube and assayed for camptothecin (*see* Methods). Hematologic toxicity was defined as a wbc of less than 5000 cells/mm³ or a platelet count of less than 100,000 platelets/mm³.

†+ = Toxicity occurred; − = toxicity did not occur; NE = not evaluable.

‡Specimen not obtained.

§Treatment discontinued. Patient had azotemia.

**Treatment discontinued. Patient had cystitis.

††Patient received six doses.

The correlation between the C_{min} values and toxicity was fair. Of the 11 patients who received 15 or 20 mg/m²/day, all five with mean 24-hour camptothecin levels of 0.5 μg/ml had toxic reactions (one of these patients received six doses and one received only four); but one of the five patients with camptothecin levels less than 0.5 μg/ml also had a toxic response. One of the four patients who received five doses of less than 15 mg/m²/day had mean plasma camptothecin levels higher than 0.5 μg/ml. This patient (No. 4) died on Day 17. The granulocyte count had fallen by 38% and the hematocrit by 14%, but hematologic toxicity (wbc less than 5000 cells/mm³ or platelet count less than 100,000 platelets/mm³) had not developed. Levels of total serum protein and serum albumin exerted no noticeable effect on either plasma camptothecin levels or toxicity. Azotemia resulted in very high plasma camptothecin levels in one patient, but toxicity could not be evaluated.

In the non-azotemic patients, of the factors measured, only urine volume seemed to have a possible influence on toxicity and plasma camptothecin levels.

DISCUSSION

The toxicity of camptothecin has been widely and unpredictably variable in the course of initial clinical evaluation. Severe toxic effects occurred even though patients with obvious renal disease were excluded. We have investigated the plasma camptothecin levels 24 hours after the administration of each dose of the daily × 5 schedule of treatment to determine whether such measurements would be of value in predicting toxicity. For clinical use, a simple, sensitive, and reliable method of determination was desirable. The method developed, although somewhat less sensitive than that published (4), involves only an extraction from acidified plasma and direct determination of intensity of fluorescence. Plasma blanks are low and the method can be used on a routine basis in any clinical laboratory with the capacity to measure fluorescence.

Plasma camptothecin levels have little relation to the dose given when the dose is in the range of 6.5–20 mg/m²/day ($r = 0.16$). Gottlieb et al (1) found 24-hour plasma camptothecin levels of 3.6, 2.7, 2.9, and 5.1 μg/ml in four patients receiving doses of 2.0, 2.5, 5.0, and 7.5 mg of camptothecin/kg of body weight, respectively. Two other patients in that study receiving doses of 10 mg/kg had 24-hour levels of 6.7 and 16.2 μg/ml.

In general, high levels of camptothecin were found in the plasma of patients who had toxic reactions and low levels were found in patients who did not; however, hematologic toxicity did develop in one patient with low levels of camptothecin. Toxicity did not develop in one patient with high levels of camptothecin. The latter patient died on Day 17, and it is, therefore, conceivable that toxicity could have developed in this patient had he lived, since marked hematologic toxicity (wbc of 1900 cells/mm³) occurred in one patient who had had a wbc of 4400 cells/mm³ on Day 15.

For a compound which becomes as highly protein bound as camptothecin, toxicity is likely to relate to free drug rather than total concentration in the plasma. Calculation of free camptothecin from the binding constant and levels of plasma albumin does not significantly alter the relative profiles of plasma camptothecin levels. The variations seen are therefore not wholly explained by variable binding with plasma protein.

Our results indicate that it is likely that factors other than simple plasma camptothecin levels are involved in determining the toxicity of the drug. In order to make the measurements clinically useful, we chose to measure only C_{min} after the administration of each dose in this study. Theoretically, the area under the plasma concentration curve, or the total time during which the concentration of the drug in the plasma exceeds a minimum effective concentration, is likely to be more reliably correlated with drug toxicity, but determination of these parameters is probably impractical for clinical purposes. Even though correlation of the toxicity with the C_{min} after each dose is imperfect, this determination is useful in detecting patients in whom the drug accumulates substantially with each successive dose, and it will alert the clinician so that he can interrupt a planned course. In addition, it is conceivable that, with the occurrence of toxicity more extreme than that observed in the present study, plasma camptothecin levels would have been considerably higher. In any case, the monitoring of plasma camptothecin levels would appear to be desirable for future trials of daily treatment with camptothecin.

The possibility that the concentration of drug in the plasma and hematologic toxicity might be related to urine volume will be discussed further in a subsequent communication.

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Immunodepressive Activity of Cycloleucine (NSC-1026) in Mice [1,2,3]

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SUMMARY

The immunodepressive activity of cycloleucine was studied in mice treated with half the LD50 dose. Cycloleucine significantly inhibited the primary immune response to sheep red blood cells and the allograft reaction. The compound was effective in reducing the number of hemolysin-producing cells when it was administered from 21 days before to 1 day after the sheep erythrocytes antigen; it was most active in mice treated on Day -1. The greatest effect against the reaction elicited by transplantation antigens of weak strength was found in mice receiving cycloleucine 24 hours after grafting.

[Cancer Chemother Rep 56:579–583, 1972]

Cycloleucine is an unnatural synthetic amino acid that is thought to act as an antagonist to the amino acid valine. Biochemical studies have revealed that this compound interferes with the in vitro and in vivo incorporation of valine into the proteins of the rat (1), and that it competitively inhibits the in vitro uptake of valine and leucine in ascites tumor cells, probably because it shares a common transport system with these amino acids (2).

Cycloleucine is active against L1210 leukemia and it inhibits the growth of Adenocarcinoma 755, 6C3HED ascites lymphosarcoma, and L4946 ascites leukemia in mice as well as Walker carcinosarcoma 256 in rats (3). In phase II study, cycloleucine was strikingly effective in a significant proportion of patients with leiomyosarcoma (4).

In 1969, Frisch (5) reported that in mice very high doses of cycloleucine prevent the synthesis of hemagglutinins and hemolysins for sheep erythrocytes by reducing the number of plaque-forming cells of the spleen.

Because of the possibility that the therapeutic effect of an antineoplastic drug may be reduced if it inhibits the host's immune response to the tumor (6), we thought it reasonable to document in more detail the immunodepressive activity of cycloleucine. The purpose of this study was to evaluate the depressive activities of this compound on the primary immune response of mice to sheep red blood cells (SRBC), and to study its effects in prolonging skin allograft survival.

MATERIALS AND METHODS

Cycloleucine was dissolved in sterile isotonic saline just prior to use and administered intraperitoneally (ip) in a volume of 0.01 ml/g of mouse body weight. Mice were treated with a single constant dose of 142 mg/kg. This dose corresponds to one half of the LD50 of cycloleucine (LD50 = 284 mg/kg; 19/20 confidence limits = 254–319 mg/kg; slope = 1.28) evaluated on the basis of the same dosage schedule (7); it was chosen in an effort to obtain results which might have clinical application. In fact, we observed that doses of cycloleucine in the range of 250–500 mg/kg (used by Frisch [5]) often caused delayed mortality 15–25 days

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[3] Cycloleucine: CAS reg. No. 52-52-8; cyclopentanecarboxylic acid, 1-amino-. Supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute.

[4] Istituto di Farmacologia dell'Università di Genova, Italy.

after administration. The 30-day-LD value of the dose used in our experiments (142 mg/kg), calculated by extrapolation from experimental data, is less than the LD1. The time of administration and the number of animals in each experiment are indicated in the tables. The following experimental models were used for evaluation of immunodepressive activity.

Immune Response of Mice to SRBC

Randomly bred female Swiss albino mice, weighing 19–23 g, were treated with a single ip injection of 142 mg/kg of cycloleucine at various times (from Day −21 to Day +2) before and after immunization with SRBC on Day 0. Sheep erythrocytes were obtained in Alserver's solution and washed three times in isotonic saline. Immunization consisted of an ip injection of 0.2 ml of a 10% suspension of washed cells. To detect the number of spleen leukocytes producing anti-SRBC antibody, IgM-hemolysin-producing cells (HPFC) were assayed by the method of Dresser and Wortis (8) 4 days after immunization (Day +4). Assays of spleen-cell suspension from each mouse were run in duplicate. The plaques were counted under a 22× magnification. Since the frequency distribution of HPFC counts (X) is not normal (8), statistics were obtained from log X transformed data. The significance level was evaluated by the "Student's 1-tailed (t)" test on the transformed data.

Blood for the hemagglutinin assay was collected 7 and 14 days after immunization and left standing overnight at 2–5 C. The serum was separated from the clot and inactivated by heating at 56 C for 30 minutes. Titers were all determined simultaneously in plastic trays (using the pooled sera from five mice for each point) by the serial twofold dilution method. Sensitivity to 0.1 M mercaptoethanol[5] was used to distinguish between IgM (mercaptoethanol-sensitive) and IgG (mercaptoethanol-resistant) antibodies. Titers are reported as the $\log_2$ of the reciprocal of the highest dilution showing definite hemagglutination (a narrow ring of red surrounding a smooth mat).

Skin Allografts in Mice

Transplants were performed according to the technique of Billingham and Medawar (9) in 3–6-month-old mice by grafting approximately 60 mm^2 of ear skin from the donor onto the back of the recipient. Mice were treated with single ip injections of cycloleucine 7 days before or 24 hours after the transplant. Dressings were removed on the sixth day, and the allografts were inspected daily.

For a test of the effect of cycloleucine on skin allograft survival across histocompatibility barriers of different strength, two histoincompatibility models were used.

In the first model, C57BL/6 male mice were used as recipients, and DBA/2 male mice were used as donors. The strains C57BL/6 ($H\text{-}2^b$) and DBA/2 ($H\text{-}2^d$) differ at the H-2 locus (a complex locus which, in the mouse, constitutes the strongest barrier) and at a relatively large number of weaker H loci (10). The complete destruction of the transplanted ear skin was taken as the end point of the graft survival.

In the second model, C57BL/6 male mice were used as donors, and C57BL/6 females were used as recipients. Males and females of this strain differ at the H-Y locus. Although the rejection due to the Y factor occurs consistently in the C57BL/6 females, it is much weaker than in the H-2-incompatible model, and usually takes place by progressive shrinkage of the grafted ear skin. To calculate the survival time, the transplanted area was measured daily with calipers, and a 50% reduction of its initial size (the area of the graft on the day the bandages were removed) was taken as the end point.

In both models, median survival times were calculated according to Litchfield (11), and differences between groups were tested for statistical significance by the Wilcoxon two-sample test (12).

RESULTS

Effect of Cycloleucine on the Mouse Primary Immune Response to SRBC

As shown in table 1, treatment with a single 142-mg/kg dose of cycloleucine, when performed from Day −21 to Day +1 (with respect to the time of administration of the antigen), produced a significant decrease in HPFC. The maximal depression occurred when the compound was given on Day −1.

A long-lasting effect of this agent is indicated by the significant inhibition of the immune

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[5] Mercaptoethanol: NSC-3723; CAS reg. No. 60-24-2.

Table 1.—IgM-hemolysin-producing cells (HPFC) in mice treated ip with cycloleucine (142 mg/kg) at various times before and after immunization with SRBC on Day 0*

Day of cycloleucine administration	Body weight change† (g)	Spleen weight (mg)	Spleen weight (mg)/body weight (g)	Nucleated cells/100 mg of spleen (×10⁶)	HPFC/spleen (× 10³)	
					Mean	Range (±1 SD)
−21	+2.4	240±91	9.7±2.2	218	374‡	210–666
Controls	+6.7	321±53	10.5±1.4	226	2199	1877–2744
−7	+0.3	128±11	5.4±0.4	184	230‡	144–365
Controls	+4.6	159±24	6.7±1.1	171	995	756–1309
−3	+1.2	111±31	4.9±0.8	149	127‡	50–317
Controls	+3.8	159±24	6.7±1.1	171	995	756–1309
−1	−0.8	99±16	5.0±0.9	138	34‡	14–85
Controls	+3.5	220±9	9.6±0.4	149	1326	783–2242
0	−1.8	97±35	5.2±0.9	128	45‡	13–157
Controls	+3.5	190±44	8.4±1.6	140	447	234–850
+1	−0.5	120±37	6.0±1.8	181	144‡	83–250
Controls	+2.7	220±9	9.6±0.4	149	1326	783–2242
+2	−1.1	133±31	6.4±0.9	141	365	262–506
Controls	+1.6	190±44	8.4±1.6	140	447	234–850

* Five animals were used for each group.

† Between the day of treatment and the day of plaquing.

‡ Significant at the 5% level or less (when compared with controls).

response observed in mice treated 21 days before inoculation with SRBC. Treatment was ineffective when performed on Day +2. Spleen weight decreased in all of the treated groups; the decrease was maximal in mice given cycloleucine on Day −1. The reduction in the number of nucleated spleen cells was roughly proportional to the reduction in spleen weight.

A difference in hemagglutinin titers of at least 3 (which could represent an inhibition of 88%) was observed on Day 7 only in mice treated 24 hours before the administration of antigen, and on Day 14 in groups given the compound on Days +1 and +2 (table 2). The pattern of reduction of mercaptoethanol-resistant antibody was similar.

Table 2.—Hemagglutinin titers in mice treated ip with cycloleucine (142 mg/kg) at various times before and after immunization with SRBC on Day 0

Day of cycloleucine administration	Hemagglutinin titer			
	Day 7		Day 14	
	Total	Mercapto-ethanol-resistant	Total	Mercapto-ethanol-resistant
Controls	9	8	11	8
−21	8	6	(Not done)	
−7	8	7	9	6
−3	7	6	9	6
−1	6	5	9	5
0	8	6	9	6
+1	8	7	9	5
+2	9	8	8	5

Effect of Cycloleucine on the Mouse Allograft Reaction

The activity of cycloleucine in prolonging the survival time of ear skin grafts in H-2-incompatible mice is shown in table 3. Regardless of when administered (7 days before or 1 day after grafting), a single dose of 142 mg/kg produced practically the same moderate significant increase in graft survival (24%–28% over controls). In contrast, when tested in mice bearing H-Y-incompatible grafts (table 4), the same dose of cycloleucine displayed significantly different effects according to the timing of its administration in relation to grafting. In fact, the increase in median survival time of grafted ear skin was 27% over that of untreated controls in mice given the compound on Day −7, and 85% over that of untreated controls in mice treated on Day +1. Moreover, two mice in the latter group retained their grafts without shrinkage for the entire 160-day test period.

TABLE 3.—Survival time of H-2-incompatible skin allografts in mice treated ip with cycloleucine (142 mg/kg)

No. of mice	Day of treatment	Skin allograft survival time (days)			Increase of ST50 over controls (%)
		ST50*	19/20 Confidence limits	S†	
26	Controls	10.56	10.13–11.03	1.078	—
10	−7	13.15	10.24–14.12	1.056	24‡
17	+1	13.53	12.31–14.86	1.150	28‡

* ST50 = time at which the end point of graft survival time (complete destruction of the graft) occurs in 50% of the test animals.
† S = slope function (according to Litchfield [11]).
‡ The time distributions of rejections are statistically different from controls in both groups at the 5% level according to the Wilcoxon two-sample test.

TABLE 4.—Survival time of H-Y-incompatible skin allografts in mice treated ip with cycloleucine (142 mg/kg)

No. of mice	Day of treatment	Skin allograft survival time (days)			Increase of ST50 over controls (%)
		ST50*	19/20 Confidence limits	S†	
42	Controls	24.16	22.97–25.42	1.120	—‡
13	−7	30.76	29.36–32.23	1.060	27‡
17	+1	44.81	39.57–50.77	1.198	85‡

* ST50 = time at which the end point of graft survival time (reduction of the graft to 50% of its original size) occurs in 50% of the test animals.
† S = slope function (according to Litchfield [11]).
‡ The time distributions of rejections are statistically different in any couple of the groups at the 5% level according to the Wilcoxon two-sample test.

DISCUSSION

Our results demonstrate that cycloleucine, given to mice in a single tolerated dose, does inhibit humoral antibody production and allograft rejection; however, the immunosuppressive action of cycloleucine is less potent than that of other antineoplastic compounds. Cyclophosphamide,[a] for example, can reduce the number of HPFC to 0.001 of control levels at doses far below the LD0 value (13).

The compound significantly decreased the plaque counts observed in the spleen 4 days after SRBC injection when it was administered from 21 days before to 1 day after the administration of the antigen (table 1). It displayed maximal activity in mice treated on Day −1, and was practically ineffective on Day +2. In contrast, serum hemagglutinin titers, determined on Days +7 and +14, showed a smaller reduction (table 2). In the light of the sequential pattern in 19S and 7S immunoglobulin production exhibited by the mouse during the primary immune response to sheep erythrocytes (14), these experimental findings indicate that cycloleucine markedly reduces the 19S activity (which is maximal in the spleen on Day +4), and is less effective in depressing 7S activity (which is maximal in the serum after Day +5). Moreover, these findings demonstrate that this compound, like busulfan (15),[7] is most inhibitory when it is administered prior to immunization. Its half-life in mice of 22 days (3) may account for the long-lasting immunodepression. These results, which corroborate those of Frisch (5), may be explained by the demonstrated (16) selective destruction of macrophages caused by cycloleucine. It is also possible that cycloleucine has a selective toxic effect on the population of progenitor cells which by a process of transformation or maturation give rise to the 19S antibody system (14).

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[a] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

The data from the allograft experiments (tables 3 and 4) indicate that cycloleucine has a distinct effect on cell-mediated immunity, particularly across minor histocompatibility barriers, such as that elicited by the H-Y locus. In this instance, cycloleucine was significantly more effective when it was administered 24 hours after grafting than 7 days before. Its marked immunodepressive effect against responses elicited by weak antigens suggests that it could effectively interfere with a possible response of the host against tumor-specific antigens.

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[7] Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate; Myleran.

Kinetics of the Reduction in Viability of Cultured L1210 Leukemia Cells Exposed to 5-Azacytidine (NSC-102816)[1,2,3]

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SUMMARY

The kinetics of the reduction in viability of cultured L1210 leukemia cells exposed to 5-azacytidine have been studied. Proliferating L1210 cells were exposed to selected concentrations of 5-azacytidine for different periods of time, and the number of cells surviving treatment was estimated by animal bioassay. The minimum effective concentration (ie, the concentration at which the viability of replicating L1210 cells is reduced by 90% after a 24-hour period of exposure) was greater than 0.1 μg/ml but less than 0.32 μg/ml. The rate of reduction in viability was concentration dependent when concentrations in the range of 1–30 μg/ml were used. Cultured L1210 cells maintained under conditions not conducive to cellular proliferation were relatively insensitive to 5-azacytidine.

[Cancer Chemother Rep 56:585–591, 1972]

5-Azacytidine, a ring analog of cytidine synthesized by Pískala and Šorm (1) and later isolated from the fermentation beer of *Streptoverticillium ladakanus* by Hanka et al, has antimicrobial (2) and antineoplastic activity. It is active against AKR and L1210 murine leukemias (2,3) and Ehrlich ascites tumor (4).

5-Azacytidine has multiple effects on mammalian tissues. It is phosphorylated and incorporated into the RNA of several types of cells (5–10), thereby inhibiting RNA synthesis and interfering with many metabolic processes (5, 6, 8, 11). 5-Azacytidine inhibits the decarboxylation of orotidylic acid and de novo pyrimidine synthesis in AKR mice (12). It inhibits DNA synthesis in primary rhesus monkey kidney cells in tissue culture (13), and is incorporated into both RNA and DNA fractions in L1210 cells in culture (14).

Since 5-azacytidine has many effects on mammalian tissues and since it is incorporated into the RNA and DNA fractions of cultured L1210 cells, the present study was undertaken in an attempt to define the kinetic characteristics of cell killing when proliferating L1210 cells are exposed to lethal concentrations of 5-azacytidine. The sensitivity of cultured L1210 cells maintained under conditions not conducive to cellular proliferation and exposed to 5-azacytidine was studied also.

MATERIALS AND METHODS

Stock cultures of L1210 cells were grown and maintained in vitro as previously described (15). Cultures were started at a density of approximately 2×10^5 cells/ml; they were used

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[2] Supported by contract PH43-65-594 from Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health.

[3] 5-Azacytidine: CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-. Supplied by Drug Research and Development, DCT, NCI, and by Dr. Gary Neil, The Upjohn Co., Kalamazoo, Mich.

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[5] The authors wish to thank Dr. Robert F. Pittillo for the microbiologic assays of 5-azacytidine. We express our appreciation to Dr. H. E. Skipper and Dr. F. M. Schabel, Jr., for their helpful discussions and encouragement. We also wish to thank Mrs. Geraldine Sheffield for her excellent technical assistance and Mrs. Mary Trader for her supervision of the bioassay work.

for drug studies when the population densities were 4–5 × 10^5 cells/ml.

A detailed description of the experimental methods used in determining the kinetic effects of potential antitumor agents on the proliferating cells in cultured L1210 populations has been published (16). In general, proliferating L1210 cells are exposed to selected concentrations of drug for different periods of time, and the number of cells surviving drug treatment at each time interval is estimated by bioassay in BDF_1 (C57BL female × DBA/2 male) mice (15).

The procedure used to obtain nonproliferating cultured L1210 cells has been described recently (17). Briefly, cells from a proliferating L1210 culture are harvested by centrifugation (250 × *g*) and resuspended at the original cell density (5–8 × 10^5 cells/ml) in Earle's salt solution (18). The total number of cells remains unchanged, but the number of viable cells (determined by bioassay in BDF_1 mice) decreases exponentially (17).

To study the kinetics of the reduction in the viability of cultured L1210 cells maintained under the conditions described previously, suspensions of L1210 cells in Earle's salt solution were exposed to selected concentrations of 5-azacytidine for different periods of time. At each time interval, samples were removed from the suspensions, and the cell viability was determined by bioassay (15). Parallel suspensions of cells in Earle's salt solution without 5-azacytidine were used as controls. The cell population half-life periods were calculated from the data (17). The results are expressed as the ratio of the half-life of the control cell population to the half-life of the treated cell population.

The basis for estimating the approximate number of leukemia cells surviving a given drug dose from lifespan and survival data has been presented by Skipper et al (19). The experimental basis for the assumptions that drug treatment of a cell population usually does not induce or select cells with generation times greatly different from those of an untreated population, and that the surviving cells begin to proliferate at the normal rate immediately after implantation in mice, has been presented (20,21).

The number of surviving cells as a function of time has been described by an exponential or Gompertz function (22). First-order kinetics is characterized by a constant proportionality factor (ie, the rate of change of a quantity is proportional to that quantity), and can be expressed formally as

$$N = N_0 e^{-kt},$$

where N is the number of viable cells at any time t, N_0 is the initial number of viable cells, and k is the proportionality factor known as the first-order rate constant. If the proportionality factor also decreases by a first-order process, ie, $-dk/dt = \alpha k$, then the Gompertz function can be used to describe the kinetics (22):

$$N = N_0 e^{-(1-e^{-\alpha t})\beta/\alpha}$$

where β is the initial value of k. The extent of deviation from first-order kinetics is determined by the value of α. When α is zero, the proportionality factor k is constant, and first-order kinetics results. The basis for the choice of the mathematical form of the kinetics was provided by statistical analysis of the experimental data and has been described (23). Each set of data was analyzed with the aid of an IBM 1130 digital computing system, and best-fit curves were prepared by an IBM 1627 plotter.

The maximum 5-azacytidine concentration used in these experiments was selected in an attempt to match the theoretical maximum concentration which can be attained in the total unbound body water of mice after the administration of a single-dose LD10 of the drug, assuming that distribution in all body fluids is rapid (24). This theoretical concentration is approximately 126 μg/ml.

Hydrolysis of 5-azacytidine in neutral and basic media leads to the formation of the following products: 1-β-D-ribofuranosyl-3-guanylurea, guanidine, α-D-ribofuro[1′,2′:4,5]-2-oxazolidione, and D-ribose (25). In acidic media, hydrolysis leads to the formation of 5-azacytosine, 5-azauracil, and D-ribose (25). A 1.23 × 10^{-4} M solution of 5-azacytidine was prepared in phosphate-buffered saline (pH 7.0) and the absorbance with time was measured at 240 nm in a Gilford recording spectrophotometer thermostated at 37 C. One-cm quartz cells were used for both the 5-azacytidine solutions and the phosphate-buffer blank. The 5-azacytidine solution was prepared immediately before the absorbance was measured. The time dependence

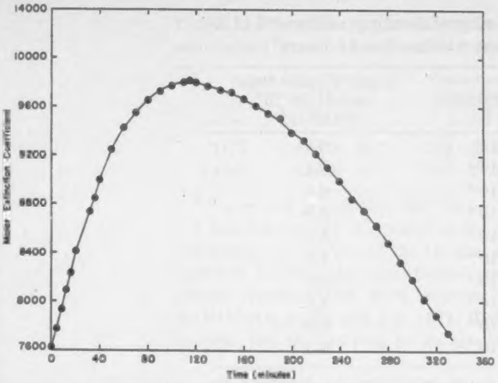

FIGURE 1.—Nominal molar extinction coefficient vs time of a 5-azacytidine solution (initial concentration, 1.23 × 10^{-4} M) in phosphate-buffered saline (pH 7.0) at 37 C.

of the molar absorptivity of the 5-azacytidine solution (fig 1) was essentially the same as that reported by Pithová et al (25).

Evaluation of the cytotoxicity of certain of the products resulting from the hydrolysis of 5-azacytidine indicated that they were considerably less active than 5-azacytidine (25). For instance, 100 μg/ml of 1-β-D-ribofuranosyl-3-guanylurea or of guanidine was required to inhibit the growth of *Escherichia coli* by 50%, whereas only 0.25 μg/ml of 5-azacytidine was required to produce the same effect (25). Microbiologic assays with *E. coli* ATCC 9637/CPU (26) indicate that the half-life of 5-azacytidine or its biologic equivalents or both is approximately 24 hours in the L1210 culture systems (Pittillo, R.F., personal communication).

RESULTS AND DISCUSSION

The increase in molar absorptivity of the 5-azacytidine solution (pH 7.0, 37 C) reaches a maximum after about 120 minutes and then begins to decrease (fig 1). This has been explained by Pithová et al (25) by the formation of an intermediate chemical substance. Although they could not characterize this intermediate, they postulated that it was the *N*-formyl derivative of 1-β-D-ribofuranosyl-3-guanylurea and presented evidence that this compound should have strong ultraviolet absorption. The kinetics of the disappearance of 5-azacytidine suggest that the intermediate is in equilibrium with 5-azacytidine but decomposes irreversibly to products which are relatively nontoxic to *E. coli*. It seems clear from figure 1 and from a figure similar to it presented by Pithová et al (25) that the concentration of this intermediate is maximal only for the first 5–6 hours of the hydrolysis. Figure 2 indicates that the killing of L1210 cells by 5-azacytidine is essentially complete after 6 hours of exposure. It may be that the cytotoxic effect and other effects observed for 5-azacytidine are mediated through the formation of this intermediate.

Pithová et al (25) also followed the disappearance of 5-azacytidine at pH 7.2 and 37 C for 36 hours by paper chromatography. They found that at this pH there was an initial rapid decrease in the concentration of 5-azacytidine, but after 6–8 hours the decline was much slower. After 24 hours, approximately 25% of the original 5-azacytidine remained. A similar result (ie, an initial rapid decline in the concentration of 5-azacytidine) was obtained by microbiologic assay of 5-azacytidine in buffered saline and the L1210 cell culture medium; however, after 24 hours, the solution retained approximately 50% of its original cytotoxicity. The quantitative discrepancy at 24 hours between the chemical analysis (25) and the microbiologic assay of 5-azacytidine must be due, at least in part, to the fact that the microbiologic assay detects the cytotoxicity of all chemical substances in the solution assayed.

Proliferating L1210 cells were exposed to a series of 5-azacytidine concentrations for 24 hours (table 1). Concentrations of 0.01 or 0.032 μg/ml were essentially nonlethal. At concentrations of 1 μg/ml or more, the reduction in the viability of the populations at 24 hours was 3–4 $\log_{10}$. The minimum effective concentration of 5-azacytidine as defined in table 1 is greater than 0.1 μg/ml but less than 0.32 μg/ml. Li et al (14) have reported that 0.15 μg/ml of 5-azacytidine inhibited the growth of L1210 cells in culture by 90%.

The kinetics of the reduction in the viability of replicating L1210 cells exposed to lethal concentrations of 5-azacytidine deviated from first-order and could be described by a Gompertz function (fig 2). The initial rate of reduction in viability (β) was concentration dependent, at least for concentrations of up to 30 μg/ml. The maximum degree of cell killing was

TABLE 1.—Reduction in the viability of proliferating cultured L1210 leukemia cells exposed to 5-azacytidine for 24 hours*

Concentration of 5-azacytidine (μg/ml)	Surviving fraction of cells †	Approximate $\log_{10}$ reduction in viability‡
0.01	1.0×10^{0}	0
0.032	7.6×10^{-1}	0.1
0.1 §	2.3×10^{-1}	0.6
0.32 §	7.0×10^{-3}	2.2
1.0	7.0×10^{-4}	3.2
3.2	1.0×10^{-4}	4.0
10.0	9.0×10^{-5}	4.0
32.0	1.0×10^{-4}	4.0
100.0	8.0×10^{-5}	4.0
200.0	5.0×10^{-5}	4.3

* Number of cells surviving at 24 hours estimated by bioassay in BDF_1 mice (19).

† Surviving fraction = N/N_0, where N_0 is initial number of viable cells present in the populations before addition of 5-azacytidine and N is number of viable cells at 24 hours.

‡ Approximate $\log_{10}$ reduction in viability = $\log_{10}$ (1/surviving fraction).

§ Minimum effective concentration of a drug is defined as that concentration which will produce a 90% reduction in the viability of proliferating cultured L1210 cells when the period of exposure to that agent is 24 hours. Based on this definition, the minimum effective concentration for 5-azacytidine is greater than 0.1 μg/ml but less than 0.32 μg/ml.

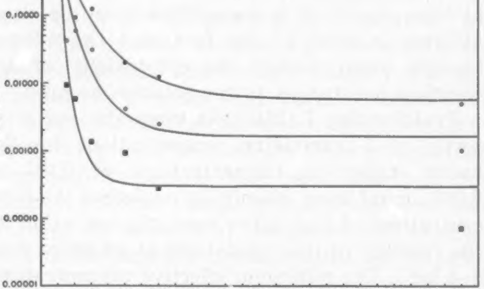

FIGURE 2.—Rates of reduction in viability of proliferating cultured L1210 leukemia cells exposed to 5-azacytidine. Kinetic plots shown are for concentrations of 1.17 (●—●—●), 11.7 (○—○—○), and 117.1 μg/ml (■—■—■). Kinetic parameters at each concentration used were:

μg/ml	β ± SE (min^{-1} × 10^3)	α ± SE (min^{-1} × 10^3)
1.17	30 ± 8	6 ± 2
1.87	28 ± 10	4 ± 2
11.7	60 ± 29	9 ± 4
30.0	194 ± 46	27 ± 6
117.1	157 ± 64	19 ± 7

achieved rapidly, ie, within approximately 360 minutes at concentrations of 1.17 or 11.7 μg/ml, and within approximately 180 minutes at a concentration of 117.1 μg/ml.

L1210 cells maintained in culture under conditions not conducive to proliferation were relatively insensitive to 5-azacytidine (table 2). Hydroxyurea, cytosine arabinoside, and 5-hydroxypicolinaldehyde thiosemicarbazone,[6] agents which interfere primarily with DNA synthesis, also do not affect the viability of L1210 cells maintained under these conditions (17,27). On the other hand, BCNU,[7] a cell cycle-nonspecific agent, will kill L1210 cells held under conditions not conducive to cellular proliferation (27).

Recently, Wilkoff et al (17) published data which indicated that proliferating cultured L1210 cells exposed to a lethal dose of tritiated

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[6] Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1. Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C. 5-Hydroxypicolinaldehyde thiosemicarbazone: NSC-107392; CAS reg. No. 19494-89-4.

[7] BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

TABLE 2.—Sensitivity to 5-azacytidine of cultured L1210 leukemia cells maintained under conditions not conducive to cellular proliferation

Concentration of 5-azacytidine		Treated cell population $T_{1/2}$ ± SE* (mins)	Control cell population $T_{1/2}$ ± SE* (mins)	Control cell population $T_{1/2}$/treated cell population $T_{1/2}$†
μg/ml	Mols/liter			
11.7	4.8×10^{-5}	154 ± 61	207 ± 29	1.3
117.0	4.8×10^{-4}	136 ± 57	207 ± 29	1.5

* $T_{1/2}$ = cell population half-life.

† The results are expressed as the ratio of the $T_{1/2}$ of the control cell population to the $T_{1/2}$ of the treated cell population. In contrast, when cultured L1210 cells maintained under conditions not conducive to cellular proliferation were exposed to 1.0 μg/ml (4.7×10^{-6} M) of BCNU, this ratio was 3.5 (17). This agent is considered to be nonspecific, and in fact may be an alkylating agent (24,34).

thymidine (H^3-TdR) were killed at a relatively rapid rate over the first 360 minutes of exposure. Since the observed reduction in the viability of the population at 360 minutes was approximately 3.0 $\log_{10}$, this was interpreted as indicating that about 99.9% of the cell population had been in S phase or had passed into S phase during the first 360 minutes of exposure, and that for approximately 0.1% of the cells in the population the duration of $G_2 + M + G_1$ was more than 360 minutes (17). The rapid rates of reduction in the viability of proliferating L1210 cells over the first 180–360 minutes of exposure to 5-azacytidine (fig 2) and the known incorporation of 5-azacytidine into DNA (to be discussed) suggest that L1210 cells in S phase are the most sensitive to 5-azacytidine. Evidence that Chinese hamster fibroblast cells in S phase are the most sensitive to 5-azacytidine has been presented recently by Li et al (28).

The mechanism of action of 5-azacytidine has, as yet, not been elucidated. The primary lethal lesion produced by 5-azacytidine may be due to interference with the sequence of events in DNA synthesis (14,28). A portion of 5-azacytidine (10%–20%) is incorporated into the DNA of L1210 cells; thus, this agent causes considerable chromosomal damage when cultured L1210 cells are exposed to it (14). The effects of 5-azacytidine on crude preparations of thymidine kinase, cytidine kinase, ribonucleotide reductase, and DNA polymerase could not be detected, and it has been suggested that inhibition of DNA synthesis by 5-azacytidine is probably the result of the incorporation of the agent into DNA and the subsequent prevention of further synthesis (14). The significance of the other effects of 5-azacytidine on metabolism (ie, incorporation into RNA, and interference with RNA and protein synthesis) in relation to lethal lesions also remains to be determined.

The primary lesion produced in DNA synthesis by cytosine arabinoside is interference with DNA polymerase (29); the primary lesion produced in DNA synthesis by hydroxyurea is interference with ribonucleotide reductase (30). Proliferating cultured L1210 cells exposed to lethal concentrations of hydroxyurea or cytosine arabinoside are killed at a rate which is independent of drug concentration over a wide range of concentrations. For instance, the rates of reduction in the viability of replicating L1210 cells exposed to hydroxyurea were essentially the same at concentrations between 125 and 8000 μg/ml (23). The rates of reduction in the viability of replicating cultured L1210 cells exposed to cytosine arabinoside (3.2–3000 μg/ml) were relatively independent of the concentration used (17). At lower concentrations, the rates of reduction are concentration dependent.

The rates of reduction in the viability of proliferating L1210 cells exposed to 5-azacytidine are concentration dependent at concentrations between 1 and 30 μg/ml. This concentration range is a relatively large fraction of the corresponding LD10 of 5-azacytidine in mice, but, for hydroxyurea and cytosine arabinoside, the concentration-independent range of cell killing is a relatively small fraction of the corresponding LD10.

Schabel (31) showed that hydroxyurea is much more effective against L1210 leukemia implanted intraperitoneally in mice when it is given every 3 hours for 24 hours on Days 2, 6, 10, and 14 after tumor implantation, than when it is given once daily. Recent studies have shown that the same total dose of cytosine arabinoside, given every 3 hours for 24 hours 2, 6, 10, and 14 days after implantation of L1210 leukemia in miçe, resulted in a greater degree of reduction in the viability of in vivo cell populations than did the same total dose administered once daily for 15 days (32). Venditti (33) has recently published data showing that 5-azacytidine has no important schedule dependency: daily and intermittent treatment of mice bearing L1210 leukemia were equally effective and superior to treatment on Day 1 only.

The data presented in this report indicate that 5-azacytidine is a cell cycle-specific agent since nonproliferating cultured L1210 cells are relatively insensitive to concentrations of 5-azacytidine which markedly reduce the viability of proliferating cultured L1210 cells. The data presented also indicate that 5-azacytidine is probably an S-phase-specific agent but that there are some striking differences between 5-azacytidine and some other commonly known agents of this class, ie, hydroxyurea and cytosine arabinoside. Cell killing is concentration dependent over a relatively wide range of concentrations (1–30 μg/ml), and there is no important schedule dependency. These differences possibly indicate that the action of 5-azacytidine is not strictly limited to the S phase; however, the data indicate that effective in vivo therapy can be enhanced by increasing doses within the limits of toxicity to normal tissues.

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Pharmacologic and Antitumor Effects of 1,2:5,6-Dianhydrogalactitol (NSC-132313) [1]

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SUMMARY

1,2:5,6-Dianhydrogalactitol produced a marked inhibition of tumor growth in five mouse tumors (NK/Ly ascites tumor, L1210 leukemia, S180 sarcoma, Ehrlich ascites tumor, and Harding-Passey melanoma) and in five rat tumors (Walker carcinosarcoma 256, Yoshida sarcoma, Guérin carcinoma, induced rhabdomyosarcoma, and a DMBA-induced myelocytic leukemia). It also produced increases in survival time of up to 200% in some of these tumor systems. Because of its histologic and cytologic effects, dianhydrogalactitol can be considered an alkylating agent. The drug exerts a severe and fast-acting hematologic effect by decreasing both the myeloid and the lymphoid cell levels, though the latter cells were more affected. Comparison studies showed that dianhydrogalactitol differs from dibromodulcitol in its toxic, antitumor, and hematologic effects.

[Cancer Chemother Rep 56:593–602, 1972]

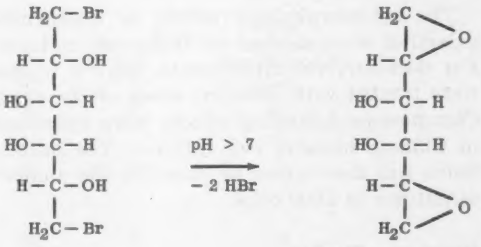
The diepoxy derivatives formed in alkaline medium from the known cytostatic dibromohexitols are the possible cause of the antitumor activity of these terminally substituted hexitols (1,2). 1,2:5,6-Dianhydrogalactitol (fig 1) is the most chemically stable of all the diepoxyhexitols, and it exerts the most pronounced biologic effect (3). To compare this substance with dibromodulcitol [4] we performed the detailed investigations which are presented in this report.

MATERIALS AND METHODS

Dianhydrogalactitol, which is highly soluble, was synthesized by L. Institóris at the Chinoin Pharmaceutical and Chemical Works, Ltd., Budapest, Hungary (1), according to the method of W. C. J. Ross (2). It was dissolved in isotonic saline and was administered intravenously (iv) or intraperitoneally (ip). In distilled water the solution had a slight, locally irritating effect. Dibromodulcitol is hardly soluble in water, therefore a 0.5% carboxymethylcellulose-containing homogenate was prepared and injected either iv or ip.

Animals

For L1210 mouse leukemia studies, first generation hybrids of BDF_1 mice weighing 20–22



Dibromodulcitol

Dianhydrogalactitol

FIGURE 1.—Chemical structures of dianhydrogalactitol (mol wt = 146) and dibromodulcitol (mol wt = 308).

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[1] Received Apr 26, 1972; revised Aug 3, 1972.

[2] Research Institute of Oncopathology, Budapest, Hungary.

[3] Chinoin Pharmaceutical and Chemical Works, Ltd., Budapest, Hungary.

[4] Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-.

g were used; for the other mouse tumor studies, outbred Swiss mice weighing 25–30 g were used. Rat tumors were maintained in Wistar/Cbi outbred and WOP inbred rats weighing 150–200 g.

Acute Toxicity Experiments

After a single dose of either dianhydrogalactitol (12–20 mg/kg) or dibromodulcitol (70–1000 mg/kg) had been given to mice the survivors were observed for 21 days. The LD50s were determined by the method of Litchfield and Wilcoxon (4). The toxic accumulations of drug were calculated from the formula (5):

$$\frac{100\ (d_3 - d_2) - d_4}{d_1 - d_4} = \%\ \text{residue},$$

where d_1 = number of deaths after a dose of 18 mg/kg (LD80); d_2 = number of deaths after a dose of 9 mg/kg (one half the LD80); d_3 = number of deaths after two doses, each 9 mg/kg (one half the LD80); and d_4 = number of deaths among the control animals.

Chronic Toxicity Experiments

In the chronic toxicity experiments rabbits were treated with dianhydrogalactitol given iv at two different dose levels every second day for 30 or 70 days. The dose levels were 2.5 and 0.25 mg/kg. The animals were killed after the 15th treatment (in animals treated for 30 days) and after the 35th treatment (in animals treated for 70 days). Every 2 weeks blood cell counts were done, and at the end of treatment histologic evaluations of all organs were performed.

Tissue Culture Studies

The cytomorphologic effects of dianhydrogalactitol were studied on HeLa cell cultures. For dose-survival experiments, Barle's L cells were treated with different doses of the drug. Chromosome-damaging effects were examined in Chinese hamster cell cultures. The mitotic index was determined by counting the number of mitoses in 1000 cells.

Histologic Studies

The sequential pattern of morphologic changes was studied in rats that had received subcutaneous (sc) tumor implantation with Guérin carcinoma cells when they were 14 days old. The studies were carried out from 1 hour to 21 days after the rats had received either a single 10-mg/kg dose (LD50), a single 5-mg/kg dose (one half the LD50), or a single 2.5-mg/kg dose (one fourth the LD50) of dianhydrogalactitol. After the treatment the animals were killed and histopathologic and cytologic alterations were investigated in the tumor, liver, spleen, kidneys, adrenal glands, duodenum, lymph nodes, and bone marrow. The alterations observed in these rats were compared to those observed in the rabbits used in the chronic toxicity tests.

Electron Microscopic Studies

A single dose of 3.7 mg/kg (one fourth the LD50) or 7.5 mg/kg (one half the LD50) of dianhydrogalactitol was administered ip to mice bearing the Ehrlich ascites tumor 6 days after tumor transplantation. The animals were killed successively 1–144 hours after the treatment. The ascitic fluid was fixed in 2.5% glutaraldehyde and 1% osmiumtetroxide according to previously used methods (7). The material was embedded in Durcupan (ACM Fluka). The ultra-thin sections were contrasted with lead citrate. These investigations were repeated once.

Hematologic Studies

The hematologic effects of dianhydrogalactitol were studied in one group of rabbits after single 14.0-mg/kg doses (LD50) and in additional groups of rabbits after 17 × 2.5- and 17 × 0.25-mg/kg doses (four rabbits per dose level). All doses were administered iv. The blood cell counts and smears were studied for 4 weeks.

Antitumor and Survival Time Studies

Five strains of mouse tumors and five strains of rat tumors were used for the evaluation of tumor response and increase in lifespan. For studies in L1210 leukemia, 10^6 ascitic or spleen cells were transplanted ip or sc respectively. Treatment was started 24 hours (early) or, in a few experiments, 4 days (advanced) after tumor transplantation. The NK/Ly and Ehrlich ascites tumors were transplanted ip using 5×10^6 cells. The rat tumors were transplanted iv using $0.5–3.0 \times 10^6$ cells. In studies of tumor growth inhibition, the animals were killed 8–15 days after tumor transplantation and the tumors were measured. In studies of increases in lifespan, observations were stopped when the treated animals had survived four times as long as the control animals.

RESULTS

Toxicity Studies

In the animals treated with the LD50 of dianhydrogalactitol (15 mg/kg), the gastrointestinal symptoms (including weight loss) were dominant. These symptoms are characteristic of alkylating agents. Atrophy of the spleen and lymph nodes was more marked after treatment with dianhydrogalactitol than after treatment with dibromodulcitol.

The animals treated with dianhydrogalactitol died earlier than those treated with dibromodulcitol. The acute toxicity values for dianhydrogalactitol and dibromodulcitol are shown in table 1.

The toxic accumulations of dianhydrogalactitol were small. Forty-eight hours after the start of treatment 45% of the drug was accumulated, and 96 hours after the start of treatment no drug was detectable (table 2).

These and previous studies (5) have shown that 5 days after the start of treatment the drug accumulation of five various cytostatic hexitol derivatives is 0 for dianhydrogalactitol; 62% for dibromodulcitol; 20% for dibromomannitol; 25% for mannitol mustard; and 45% for mannitol myleran.[5]

In chronic toxicity experiments in rabbits

TABLE 1.—Acute toxicity values for dianhydrogalactitol and dibromodulcitol (10 animals per dose level) (6)

Animal	Route of administration	LD50 (mg/kg) Dianhydrogalactitol	LD50 (mg/kg) Dibromodulcitol
Mouse	ip	15.0	1000
	iv	20.5	—
	oral	16.5	—
Rat	ip	11.2 (10.2–12.3)	470
	iv	16.0–18.0	—
	oral	14.0–16.0	1000
Rabbit	iv	14.0	—
	oral	—	300

TABLE 2.—Toxic accumulation of dianhydrogalactitol in mice

Group No. (10 animals/group)	Dose (ip injection)	Day of injection	Factor	Mortality rate (%)	Toxic accumulation after 96 hours* (%)
A	18 mg/kg (LD80)	Day 1	d_1	80	67
B	9 mg/kg (one half the LD80)	Day 1	d_2	10	0
C	9 mg/kg (one half the LD80)	Days 1 and 4	d_3	30	0
D	Control	—	d_4	0	0

*Accumulation $= \frac{100\ (d_3 - d_2) - d_4}{d_1 - d_4} = \frac{0}{67} = 0.$

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[5] Dibromomannitol: NSC–94100; CAS reg. No. 488-41-5; 1,6-dibromo-1,6-dideoxy-D-mannitol.

Mannitol mustard: NSC–9698; CAS reg. No. 551-74-6; mannitol, 1,6-bis[(2-chloroethyl)amino]-1,6-dideoxy-, dihydrochloride, D-.

Mannitol myleran; NSC–37538; CAS reg. No. 1187-00-4; mannitol, 1,6-dimethanesulfonate, D-.

the most marked alterations were cell population reductions (especially of the myeloid elements) in the bone marrow. Decreased blast cell counts and some cells with pyknotic nuclei were observed. No severe toxic changes of the organs were noted.

Tissue Culture Studies

The mitotic index of untreated control HeLa cultures was 2.9%. A 10-μg/ml dose of dibromodulcitol decreased the mitotic index to 0, and a 100-μg/ml dose of dianhydrogalactitol caused complete cellular disintegration. Dose-survival curves obtained from dianhydrogalactitol- and dibromodulcitol-treated L cells are illustrated in figures 2 and 3 (8). The cloning efficiency of untreated cells varied between 60% and 80%. The cloning efficiency of treated cells is expressed in percent of 100% control values. The curve of dianhydrogalactitol-treated cultures is rather steep between 1 and 10 μg/ml. In the case of dibromodulcitol, only the first part of the curve is steep; it then becomes less steep and linear. Fifty percent survival was obtained when 4 μg/ml of dianhydrogalactitol or 75 μg/ml of dibromodulcitol was given.

The cytomorphologic effects of dianhydrogalactitol were characterized by various mitotic abnormalities, such as irregular arrangement of chromosomes, frequently resembling multipolar mitosis. In heavily damaged cells, chromosomes were clumped into a dense mass and extrachromosomal parts contained granules. Forty-eight hours after treatment cells were considerably enlarged, and 24 hours later multinucleate cells were found.

Low doses of dianhydrogalactitol induced characteristic chromosome aberrations in a number of Chinese hamster cells in vitro, eg, chromatid and isochromatid breaks and gaps, acentric and dicentric chromosomes, and a wide range of symmetric and asymmetric exchanges. Chromosome fragmentation showed heavy cell damage. Chromosomal translocations were frequently noted.

Histologic Studies

Mitotic alterations appeared soon after the start of treatment with dianhydrogalactitol and were long lasting. This was demonstrated in all organs, but especially in the tumor, where, due to small doses, there was an increase in malformed mitoses and a large number of pyknomitoses.

Severe alterations were observed in an increasing number of mitoses 24–48 hours after the start of treatment. The most marked effect appeared after 72–96 hours when every second cell was dividing (fig 4). During the early mitotic alterations no polyploid cells were present; however, later there were giant cells with a large number of chromosomes. There was an

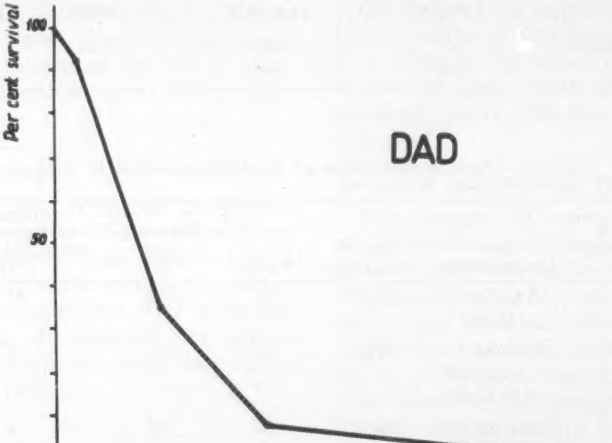

FIGURE 2.—Dose-survival curve for dianhydrogalactitol (DAD) in asynchronously growing L cells. Exposure time = 1 hour.

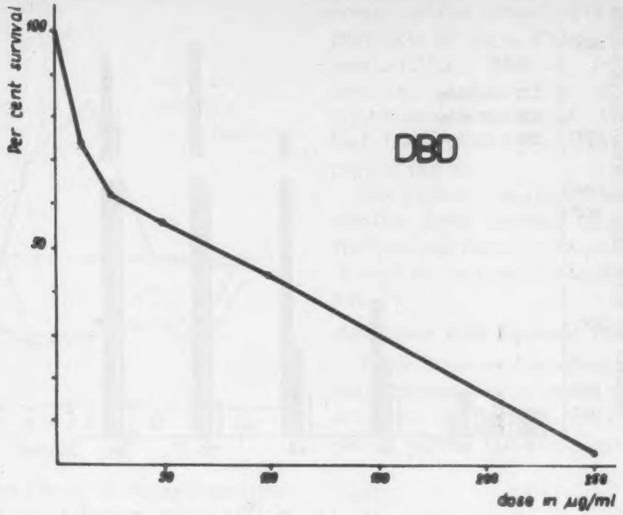

FIGURE 3.—Dose-survival curve for dibromodulcitol (DBD) in asynchronously growing L cells. Exposure time = 1 hour.

increase in the number of giant cells after treatment with dianhydrogalactitol, but this increase was not as large as in the case of treatment with mannitol mustard or dibromodulcitol (fig 5). Along with the increase in the number of giant cells the necrotic areas increased but never to an extreme degree. Expressed desmoplastic activity was not observed.

Soon after the start of treatment pyknotic cell decay dominated the duodenum followed by reticulohistiocytic phagocytosis of the debris. First, the goblet cells and the mitoses increased in the crypts and tubules; later, the mitotic figures suddenly disappeared. The marked lymphoid infiltration altered the structure of the duodenum, and the villi were flattened and deformed.

There was a reduction in the number of myeloid cells in the bone marrow. There was an increase in the number of reticular cells, and dilated sinusoids were filled with blood. Pyknotic changes appeared on the nuclei of the mega-

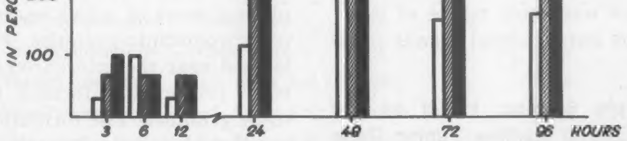

FIGURE 4.—Histologic alterations (number of mitoses) in Guérin carcinoma cells caused by treatment with dianhydrogalactitol (DAD).

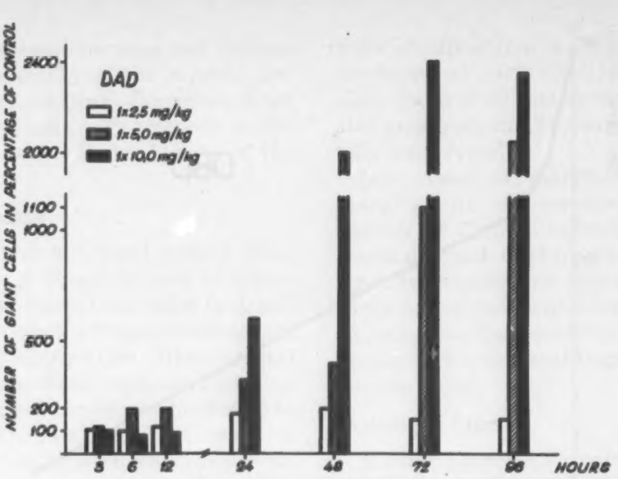

FIGURE 5.—Histologic alterations (number of giant cells) in Guérin carcinoma cells caused by treatment with dianhydrogalactitol (DAD).

karyocytes. Diffuse repopulation occurred and later (in 72–96 hours) it became focal.

The early drug response in the spleen resulted in a decay of the lymphoid cells in the malpighian follicles. The phagocytosis of the debris by reticulum cells occurred first at the follicles and later in the pulp. Soon after treatment (6–12 hours) the lining cells of the sinusoids were destroyed, and the pulp became poorly populated and hyperemic; a few plasma cells, myeloid cells, and swollen, pigment-containing reticulum cells appeared. After larger doses or longer times from the start of treatment (24–48 hours) the pulp became empty and the reticular cells were prominent. Soon after this, repopulation started.

In the lymph nodes, the alterations were similar to those of the spleen. Three hours after treatment cell decay appeared in the follicles and phagocytosis of the debris by reticulum cells occurred. The process was short-lived and after 48 hours there were only traces of it.

No changes in the parenchymal organs could be observed.

Electron Microscopic Studies: Effect on the Ultrastructure of Ehrlich Ascites Tumor Cells

The effects of single 3.7- and 7.5-mg/kg doses of dianhydrogalactitol at the ultrastructural level were studied in animals bearing the Ehrlich ascites tumor. Treatment with larger doses proved to be more effective.

The nucleus and cytoplasm were damaged by treatment with the larger dose of the drug. We could detect the earliest effects 3 hours after administration. The matrix of the mitochondria disappeared and the cristae and mitochondrial membranes were fragmented. A more marked effect could be seen 48–72 hours after the administration of the drug. The ribosomes were detruded from the surface of the endoplasmic reticulum, and aggregates were formed by the free ribosomes. The small vesiculi of the smooth endoplasmic reticulum increased.

After 3 hours, various nuclear alterations occurred; the chromatin substance was loosened and decreased, the compact nucleoli disappeared, and a marked electron-dense spottedness could be observed (9). Eight to ten electron-dense granules (0.1μ–0.3μ in diameter) could be seen arranged in groups in the nucleoplasma, some of which exceeded the size of the perichromatinic granules. These granules were located near the interchromatinic granules and were presumably formed by the clotting of these granules. The formation of such granules was not observed after other chemotherapeutic agents had been given. Multinucleated cells were detected more infrequently after dianhydrogalactitol treatment than after treatment

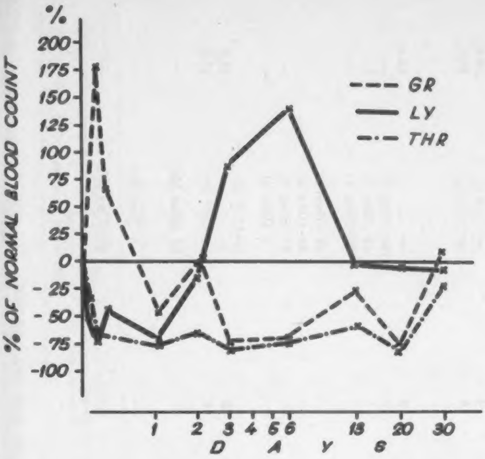

Figure 6.—Hematologic effects of dianhydrogalactitol (DAD) (14.0 mg/kg × 1 iv) on rabbits. GR = granulocyte count; LY = lymphocyte count; THR = thrombocyte count.

with other alkylating agents (mannitol mustard, dibromodulcitol).

The effect of smaller doses (3.7 mg/kg) could only be detected 72–96 hours after treatment. At this time, marked mitochondrial alterations were seen.

The effect of dianhydrogalactitol on the ultrastructure of tumor cells is reminiscent of that of other alkylating agents.

Hematologic Effects

After a single large dose (14.0 mg/kg) of dianhydrogalactitol in rabbits, marked reactive leukocytosis occurred, followed by a 50% decrease in the granulocyte count which disappeared in 30 days. The number of lymphocytes was similarly reduced, although regeneration occurred earlier (Day 13) when temporary lymphocytosis occurred. After this large dose had been given persistent thrombopenia occurred (fig 6).

The animals receiving repeated injections of smaller doses showed moderate lymphoid responses and more severe myeloid responses, although no changes in platelet counts were noted (fig 7).

Antitumor and Survival Time Studies

Table 3 shows the effect on survival time of dianhydrogalactitol given to mice with L1210 leukemia compared to the effects of other alkylating agents tested in our Institute. Various

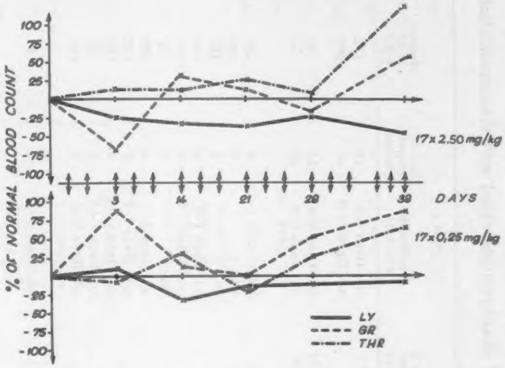

Figure 7.—Hematologic effects of dianhydrogalactitol (DAD) (2.50 mg/kg × 17 and 0.25 mg/kg × 17, 4 animals per dose level) on rabbits. LY = lymphocyte count; GR = granulocyte count; THR = thrombocyte count.

Table 3.—Effect on L1210 leukemia of dianhydrogalactitol compared with effects of different alkylating agents

Compound	Treatment schedule, ip	Optimal dose (mg/kg/day)	Median ILS (%)	No. of survivors on Day 60/total No. treated
Dianhydrogalactitol	Once, Day 1	10	200	2/6
Dianhydromannitol	Once, Day 1	25	40	0/6
Dibromodulcitol	qd, Days 1–9	300	67	0/6
Mannitol mustard	qd, Days 1–7	15	91	0/6
Dibromomannitol	qd, Days 1–6	250	13	0/6
Busulfan	Once, Day 1	20	0	0/6
Mannitol myleran	qd, Days 1–9	500	23	0/6

TABLE 4.—Antitumor effects of dianhydrogalactitol and dibromodulcitol on five mouse tumors

Tumor, route of tumor injection	Drug	No. of doses × mg/kg/dose (ip injection)	Treatment schedule	No. of animals treated	Median ILS* (%)	No. of survivors by 40–60 days after start of treatment	Percent of average tumor inhibition (day animals were killed)	Median ILS (control)
NK/Ly ascites tumor, ip	Dianhydrogalactitol	3 × 2.5†	qd, Days 1–3	10	179	0	—	16.2
		10 × 2.5	qd, Days 1–11	10	290	0	—	—
	Dibromodulcitol	6 × 125.0†	qd, Days 1–7	10	90	0	—	45.6
		6 × 150.0	qd, Days 1–7	10	—	—	56 (Day 10)	—
L1210 leukemia, ip (treatment started 24 hours after tumor transplantation)	Dianhydrogalactitol	1 × 10.0	Day 1	6	200	2‡	—	—
		1 × 10.0	Day 1	6	140	0	—	9.6
		1 × 7.5	Day 1	6	136	0	—	9.3
		2 × 7.5	qd, Days 1–2	6	132	0	—	9.8
		3 × 7.0	q4d, Days 1–9	6	184	1‡	—	—
		1 × 5.0	Day 1	6	121	0	—	9.8
		5 × 5.0	qd, Days 1–5	6	126	0	—	10.6
		3 × 4.0	q4d, Days 1–9	6	142	0	—	10.2
		3 × 2.5	qd, Days 1–3	6	138	0	—	10.3
		9 × 2.5	qd, Days 1–10	6	135	0	—	10.0
		10 × 1.0	qd, Days 1–11	6	150	0	—	10.6
L1210 leukemia, sc (treatment started 4 days after tumor transplantation)	Dianhydrogalactitol	14 × 2.5	qd, Days 4–17	6	38	0	—	15.6
L1210 leukemia, ip (treatment started 24 hours after tumor transplantation)	Dibromodulcitol	6 × 125.0	qd, Days 1–7	6	41	0	—	10.3
		2 × 600.0	qd, Days 1–2	6	41	0	—	10.2
Ehrlich ascites tumor, ip	Dianhydrogalactitol	9 × 2.5	q2d, Days 1–17	10	274	6	—	18.4
		6 × 0.75	qd, Days 1–7	10	—	—	100 (Day 8)	—
		6 × 0.50	qd, Days 1–7	10	—	—	49 (Day 8)	—
		6 × 0.25	qd, Days 1–7	10	—	—	68 (Day 8)	—
	Dibromodulcitol	9 × 200.0	q2d, Days 1–17	10	—	—	65 (Day 8)	—
		6 × 66.0	qd, Days 1–7	10	—	—	65 (Day 8)	—
		6 × 33.0	qd, Days 1–7	10	—	—	36 (Day 8)	—
		6 × 16.5	qd, Days 1–7	10	—	—	0 (Day 8)	—
S180 ascites tumor, ip	Dianhydrogalactitol	1 × 5.0	qd, Day 1	10	120	5	—	18.0
		8 × 2.5	qd, Days 1–8	10	100	4	—	18.0
	Dibromodulcitol	8 × 200.0	qd, Days 1–8	10	—	—	56 (Day 10)	—
Harding-Passey melanoma, sc	Dianhydrogalactitol	13 × 2.5	q3d, Days 1–37	6	—	—	43 (Day 38)	—
	Dibromodulcitol	13 × 200.0	q3d, Days 1–37	6	—	—	67 (Day 38)	—

*ILS = increase in lifespan.
†One eighth the LD50.
‡Results from Drug Research & Development, Division of Cancer Treatment, National Cancer Institute.

TABLE 5.—Antitumor effect of dianhydrogalactitol and dibromodulcitol on five rat tumors

Tumor, route of tumor injection	Drug	No. of doses × mg/kg/dose (ip injection)	Treatment schedule	No. of animals treated	Median ILS* (%)	Survivors by 60 days after treatment (%)	Percent of average tumor inhibition (day animals were killed)	Median ILS (control)
Walker carcinosarcoma 256, sc	Dianhydrogalactitol	5 × 1.25	qd, Days 1–5	6	—	—	100 (Day 8)	—
	Dibromodulcitol	6 × 100.0	qd, Days 1–7	6	—	—	95 (Day 8)	—
Walker carcinosarcoma 256, im	Dianhydrogalactitol	4 × 2.5	q2d, Days 1–7	6	—	—	95 (Day 8)	—
	Dibromodulcitol	4 × 200.0	q2d, Days 1–7	6	—	—	96 (Day 8)	—
Walker carcinosarcoma 256, iv	Dianhydrogalactitol	16 × 2.5	qd for 4 days, then pause for 3 days, Days 1–25	10	∞	100	—	15
	Dibromodulcitol	3 × 200.0 (orally)	qd, Days 1–3	6	150	75	—	8
Yoshida ascites tumor, ip	Dianhydrogalactitol	3 × 2.5	q2d, Days 1–5	6	195	0	—	7
	Dibromodulcitol	7 × 100.0	qd, Days 1–7	6	125	60	—	8
Yoshida sarcoma, sc	Dianhydrogalactitol	6 × 1.0	qd, Days 1–7	6	—	—	65 (Day 9)	—
		6 × 2.0	qd, Days 1–7	6	—	—	74 (Day 9)	—
		6 × 3.0	qd, Days 1–7	6	—	—	72 (Day 9)	—
	Dibromodulcitol	6 × 50.0	qd, Days 1–7	6	—	—	93 (Day 9)	—
		6 × 25.0	qd, Days 1–7	6	—	—	94 (Day 9)	—
		6 × 12.5	qd, Days 1–7	6	—	—	89 (Day 9)	—
		6 × 6.25	qd, Days 1–7	6	—	—	27 (Day 9)	—
		6 × 4.0	qd, Days 1–7	6	—	—	31 (Day 9)	—
Guérin carcinoma, sc	Dianhydrogalactitol	16 × 1.25	qd, Days 1–16	6	—	—	65 (Day 17)	—
	Dibromodulcitol	6 × 200.0	qd, Days 1–7	6	—	—	98 (Day 17)	—
Guérin carcinoma, iv	Dianhydrogalactitol	16 × 2.5	qd for 4 days, then pause for 3 days, Days 1–25	10	46	20	—	30
	Dibromodulcitol	6 × 200.0	q2d, Days 1–15	6	95	40	—	—
Rhabdomyosarcoma, im	Dianhydrogalactitol	5 × 2.5	q2d, Days 1–11	6	—	—	66 (Day 15)	—
	Dibromodulcitol	7 × 200.0	qd, Days 1–9	6	—	—	93 (Day 15)	—
DMBA-induced myeloid ascites leukemia, ip†	Dianhydrogalactitol	8 × 2.0	qd, Days 1–8	6	125	0	—	13.8
	Dibromodulcitol	8 × 175.0	qd, Days 1–8	6	86	0	—	13.8
Myeloid leukemia transplanted with spleen and liver extracts, sc†	Dianhydrogalactitol	6 × 2.5	qd, Days 1–6	5	150	30	—	32
	Dibromodulcitol	2 × 150.0	qd, Days 1–2	5	175	25	—	28

*ILS = increase in lifespan.
†Data from reference 10.

doses of dianhydrogalactitol prolonged the lifespan of animals with the early form of L1210 leukemia 50%–200%, while dibromodulcitol showed only 40% efficacy in this tumor system. Dianhydrogalactitol surpassed the effects of other known related compounds.

The treatment of NK/Ly tumor-bearing mice with dianhydrogalactitol resulted in a 200% increase in lifespan. Dibromodulcitol produced a prolongation in survival time of only 90%.

Fifty percent of the animals with Ehrlich and S180 ascites tumors recovered after treatment with dianhydrogalactitol. Small doses of the drug (0.75 mg/kg; one sixtieth of the LD50) caused a 68% tumor weight inhibition in the animals with Ehrlich ascites tumor; equitoxic doses of dibromodulcitol caused no effects on the same tumor. In Harding-Passey melanoma, dibromodulcitol was more effective than dianhydrogalactitol. Table 4 shows the results of antitumor and survival time studies in the five mouse tumors.

In contrast to the mouse tumors, dibromodulcitol proved to be more effective on the solid rat tumors than dianhydrogalactitol (table 5).

Both drugs had a marked effect on the ascitic form of the DMBA-induced myelocytic leukemia in rats and on rat leukemia injected with spleen and liver cells from leukemia-bearing animals.

DISCUSSION

Dibromomannitol and dibromodulcitol are cytostatic drugs which have characteristic activity on blood cells. In addition, they inhibit the growth of several transplantable animal tumors. The first of these drugs has proved to be a useful agent against chronic myelogenous leukemia; dibromodulcitol has been used with success in busulfan-refractory chronic myelogenous leukemia and also in the treatment of polycythemia and several solid tumors.[6]

It was demonstrated earlier that both compounds undergo various biotransformational stages in the organism, in the course of which epoxydes are formed which are partly responsible for the antitumor activity (1). Dianhydromannitol[7] (derived from dibromomannitol) and dianhydrogalactitol (derived from dibromodulcitol) have become very useful and interesting research compounds by British and Hungarian researchers. Dianhydrogalactitol has proved to be of more interest because of its stability, water-solubility, and significant biologic efficacy. In view of the favorable hematologic and antitumor activity of dianhydrogalactitol, a detailed clinical trial would be reasonable.

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[6] Busulfan: NSC–750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate; Myleran.

[7] Dianhydromannitol: NSC–133129; mannitol, 1,2:5,6-dianhydro-, D-(+)-.

In Vitro Activation of Isophosphamide (NSC-109724), a New Oxazaphosphorine, by Rat Liver Microsomes [1,2]

Larry M. Allen and Patrick J. Creaven [3,4]

SUMMARY

The activation of isophosphamide (an oxazaphosphorine analog of cyclophosphamide) by rat liver microsomes has been studied. Activation is maximal in the microsomal fraction, and is dependent on nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). It is inhibited by ethylmorphine and 2-diethylaminoethyl-2, 2-diphenylvalerate (SKF-525A). The microsomal reaction conforms to simple Michaelis-Menten kinetics. Vmax for the activation of isophosphamide is the same as that for cyclophosphamide: 5.4 μmol/g of liver/hour. The Km for the activation of isophosphamide is 19.4 millimols; the Km for cyclophosphamide is 4.0 millimols. Pretreatment with phenobarbital (55 mg/kg/day × 3) causes a 174% increase in the alkylating activity of isophosphamide and a 76% increase in the alkylating activity of cyclophosphamide. The induction of alkylating activity by phenobarbital is unaffected by the simultaneous administration of isophosphamide. Pretreatment with isophosphamide, phenobarbital, or isophosphamide plus phenobarbital does not affect the induction of microsomal RNA and nuclear DNA, but isophosphamide partially prevents the induction of microsomal protein by phenobarbital.

[Cancer Chemother Rep 56:603–610, 1972]

Isophosphamide is a new antineoplastic agent which is chemically related to cyclophosphamide.[5] Structurally, the two drugs differ in only one respect: in cyclophosphamide, both chloroethyl groups are attached to the exocyclic nitrogen; in isophosphamide, one is attached to the endocyclic nitrogen (fig 1).

Cyclophosphamide must be enzymatically activated before it will exert cytotoxic or alkylating activity (1,2). This activation occurs primarily in the liver, is mediated through the microsomal mixed-function oxidase system (2,3), and is induced by pretreatment with phenobarbital (4–7).[6] Isophosphamide has been shown to be inactive in vitro also, but when it is administered to rats, it gives rise to plasma alkylating activity. Pretreatment with phenobarbital increases the level of this activity (8). Isophosphamide competitively inhibits the microsomal activation of cyclophosphamide (9) and is more effective than cyclophosphamide in the treatment of some animal tumors (8,10). In extensive initial clinical evaluation, it has been particularly effective against small cell anaplastic (oat cell) carcinoma of the lung (10). We have, therefore, studied the activation of isophosphamide in microsomal fractions iso-

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[1] Received May 12, 1972; revised July 14, 1972.

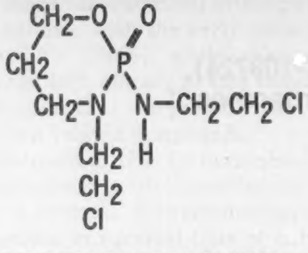
[2] Isophosphamide: CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide; Z-4942; Ifosfamide. Supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] National Cancer Institute-Veterans Administration Medical Oncology Service, VA Hospital, Wash, DC.

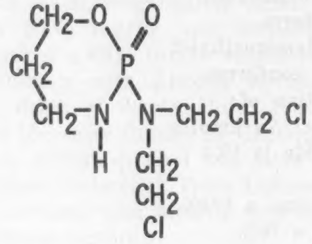
[4] The expert technical assistance of Mr. Ralph McCloud is gratefully acknowledged.

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; *2H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

[6] Phenobarbital: 5-ethyl-5-phenylbarbituric acid.



ISOPHOSPHAMIDE



CYCLOPHOSPHAMIDE

FIGURE 1.—Structures of isophosphamide and cyclophosphamide.

lated from rat liver and the induction of this activation by phenobarbital.

MATERIALS AND METHODS

All chemicals were analytical grade. Cyclophosphamide was obtained from Mead Johnson Laboratories. 2-Diethylaminoethyl-2,2-diphenylvalerate (SKF–525A) was obtained from Smith Kline & French Laboratories. Mechlorethamine,[7] calf thymus DNA, and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, bakers' yeast, 250 enzyme units [EU]/mg) were obtained from Sigma Chemical Company, St. Louis, Missouri. 4-(4-Nitrobenzyl)-pyridine (NBP) was obtained from the Eastman Kodak Co. and recrystallized with 10% ethanol. Spectrophotometric determinations were done on a Beckman DB–G spectrophotometer.

Preparation of Rat Liver Microsomes

Male Sprague-Dawley rats (150–175 g) were killed by a blow on the head, and their livers were removed and rinsed several times in ice-cold 1.15% KCl. The livers were immediately weighed, minced, and homogenized at high speed for 1 minute in a Virtis "45" homogenizer. All subsequent operations were carried out at 4 C. The liver homogenate was centrifuged at 10,000 × *g* for 20 minutes. The pellet was discarded and the supernatant was centrifuged at 105,000 × *g* for 1 hour in a No. 40 rotor of a Beckman Model L ultracentrifuge. The microsomal pellets were dispersed with a Dounce homogenizer and suspended in 1.15% KCl. One ml of suspension was equivalent to 250 mg of wet whole liver.

Incubation of Liver Homogenates and Microsomes

The standard incubation mixture consisted of: 1 ml of microsomal suspension (250 mg); 8 μmol of $MgCl_2$; 30 μmol of Na_2HPO_4–KH_2PO_4 buffer (pH 7.4); 17 μmol of glucose-6-phosphate; 0.76 μmol of nicotinamide adenine dinucleotide phosphate (NADP) (pH 7.0); 20 μl (2 EU) of glucose-6-phosphate dehydrogenase; 13.5 μmol of isophosphamide or cyclophosphamide; and 1.15% KCl to a final volume of 3.0 ml. Glucose-6-phosphate dehydrogenase was omitted from the incubation mixtures of the 1000 × *g* and 10,000 × *g* liver homogenates. Solutions of isophosphamide and cyclophosphamide were prepared immediately before they were to be used.

The standard incubation mixture in 40-ml beakers was equilibrated at 37 C in a Warner-Chillcot shaking water bath for 5 minutes and after the addition of cyclophosphamide (100 μl), isophosphamide (50 μl), or other substrates, the incubation was continued in air with shaking for 15 minutes. For cyclophosphamide and isophosphamide, the reaction was terminated by chilling the incubation mixture on ice and adding an equal volume of 95% ethanol. A tissue blank was prepared by denaturing the microsomal suspension or liver homogenate with alcohol and then adding substrate.

Pretreatment of Animals

Sprague-Dawley male rats (100 g) were allowed to have food and water ad libitum. They received phenobarbital (55 mg/kg of body weight), isophosphamide (44 mg/kg), or both, intraperitoneally (ip) daily for 3 days. Control animals received an equal volume of isotonic saline. Twenty-four hours after the administration of the last dose, the animals were killed and the microsomal fraction was prepared according to the method described previously.

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[7] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard.

Assay of Alkylating Activity

Alkylating activity was assayed by the method of Friedman and Boger (11) with a minor modification. One ml of water, 1 ml of 0.025 M sodium acetate buffer (pH 4.6), and 0.5 ml of 5% NBP in acetone were added to 1 ml of denatured sample in a 15-ml test tube. The tubes were shaken, capped with marbles, placed in a boiling water bath for 20 minutes, and then cooled very rapidly in ice; 1 ml of acetone, 3 ml of ethyl acetate, and 1 ml of 0.25N NaOH were then added. The solutions were mixed thoroughly and centrifuged in order to separate the phases. The upper phase was measured spectrophotometrically at 542 nm in a 1-cm cuvette thermoregulated at 25 C. Occasionally, the tubes had to be briefly immersed in a water bath at 30 C in order to clarify the ethyl acetate phase. The color was stable for at least 3 minutes.

The amount of degraded isophosphamide and the effect of heating on the stability of isophosphamide were determined by the method of Hohorst, Ziemann, and Brock (12). The residual alkylating activity of isophosphamide was 0.63%. After the upper phase was heated for 20 minutes at 100 C in sodium acetate buffer (0.025 M, pH 4.6), the residual alkylating activity of isophosphamide was 1.80%.

A standard curve of alkylating activity was prepared with mechlorethamine. Forty-five nanomols of mechlorethamine gave an absorbance of 0.40 by our procedure. The alkylating activity of microsomally activated cyclophosphamide or isophosphamide is expressed relative to this standard curve.

Assay of Mixed-Function Oxidases

N-demethylation of ethylmorphine and aminopyrine was assayed with double-strength Nash's reagent (13), according to the method of Davies, Gigon, and Gillette (14). The final concentrations of ethylmorphine and aminopyrine in 3 ml of incubation mixture were 9 and 13.9 millimols, respectively. Semicarbazide HCl (pH adjusted to 7.0) was added as a formaldehyde trap. The hydroxylation of aniline was assayed according to the method of Schenkman, Remmer, and Estabrook (15). The final concentration of aniline was 4.6 millimols.

Estimation of DNA, RNA, and Protein in Liver

The amount of DNA in the 10,000 × *g* pellets from the microsomal preparations was estimated as follows. The tissue was denatured with an equal volume of 20% trichloroacetic acid (TCA) and centrifuged, and the precipitate was washed (twice) successively with two volumes of each of the following: 10% TCA, 70% ethanol, ether-ethanol (1:1, v/v), and ether (all at 4 C). The prepared tissue was dried at 57 C for 30 minutes and then it was crushed to a fine powder in a mortar. Aliquots (8–10 mg) were hydrolyzed with 4 ml of 15% $HClO_4$ (PCA) at 80 C for 20 minutes. The PCA solution was diluted ten times, and the DNA was estimated by the method of Hubbard, Matthew, and Dubowik (16) using calf thymus DNA as a standard.

Aliquots from the microsomal fraction were hydrolyzed with an equal volume of 0.5N NaOH for 24 hours at 37 C and diluted 15 times, and the RNA was estimated by the method of Ceriotti (17). The microsomal protein was determined according to Lowry et al (18), using bovine serum albumin as a standard.

RESULTS

Maximal activation of isophosphamide is found with the microsomal fraction of liver and requires NADPH (reduced form of NADP). The activation is inhibited by ethylmorphine and SKF–525A, but not by aniline (table 1). The activation is linear up to 35 minutes.

The microsomal reaction conforms to simple Michaelis-Menten kinetics (fig 2). The Vmax of isophosphamide is the same as that of cyclophosphamide (5.4 μmol mechlorethamine equivalents/g of liver/hour), but the apparent Km for isophosphamide is 19.4 millimols while that for cyclophosphamide is 4.0 millimols. The effect of pretreatment with phenobarbital, isophosphamide, or both on the microsomal metabolism of isophosphamide and cyclophosphamide, the demethylation of ethylmorphine and aminopyrine, and the hydroxylation of aniline are shown in table 2. Pretreatment with phenobarbital causes a 174% increase in the alkylating activity of isophosphamide and a 76% increase

TABLE 1.—Activation of isophosphamide under different incubation conditions*

Subcellular fraction	Incubated with— NADPH	Ethylmorphine	SKF-525A	Aniline	Activation (%)
1000 × *g* supernatant	−	−	−	−	92
10,000 × *g* supernatant	−	−	−	−	74
105,000 × *g* supernatant	+	−	−	−	15
Microsomal fraction	+	−	−	−	100
Microsomal fraction	−	−	−	−	7
Microsomal fraction	+	+ (0.5 millimols)	−	−	80
Microsomal fraction	+	+ (2.5 millimols)	−	−	70
Microsomal fraction	+	−	+ (3.0 millimols)	−	78
Microsomal fraction	+	−	−	+ (4.0 millimols)	100
Microsomal fraction	+	−	−	+ (6.0 millimols)	96

* Liver fractions, incubation conditions, and methods of assay are described in the section on Methods. Each value represents the mean of at least three experiments (three animals in each experiment). Values are expressed as percent activation by microsomal fraction (with NADPH as 100%) (1.27 ± 0.29 μmol mechlorethamine equivalent/g of liver/hour).

in the alkylating activity of cyclophosphamide. Pretreatment with isophosphamide has essentially no effect on any of the activities studied and does not significantly inhibit the induction by phenobarbital. Isophosphamide pretreatment does not affect the level of liver microsomal protein, but it significantly decreases (from 56% to 20%, $P < 0.05$) the increase in microsomal protein produced by phenobarbital pretreatment. None of the pretreatments affect the level of either microsomal RNA or nuclear DNA (table 3).

Activation of isophosphamide and cyclophosphamide is not induced by the pretreatment of animals with a dose of 3-methylcholanthrene (30 mg/kg in corn oil given ip daily for 3 days), which produces a 342% increase in the hydroxylation of aniline.

DISCUSSION

The activation of isophosphamide by rat liver resembles the activation of its analog cyclophosphamide in that both are mediated by the microsomal mixed-function oxidase system, both are inhibited by type I but not by type II (19) microsomal oxidase substrates, and both are inducible by phenobarbital. The kinetics of activation are different, however. Although the Vmax is the same for both compounds, the apparent Km of isophosphamide is greater than that of cyclophosphamide. The Vmax value obtained for both compounds in the present study (5.4 μmol mechlorethamine equivalents/g of liver/hour) is similar to that reported by Sladek (6,7) for cyclophosphamide (4.2 μmol

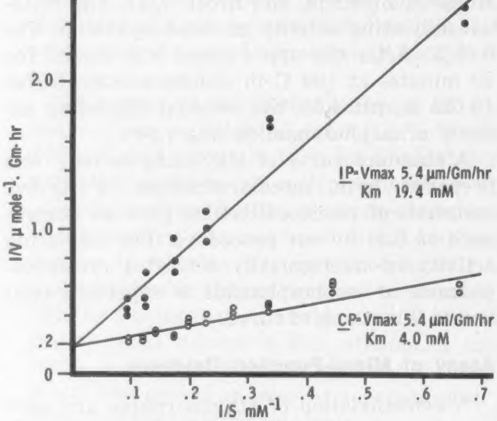

FIGURE 2.—Lineweaver-Burke plot of the activation of isophosphamide (IP) and cyclophosphamide (CP). Microsomal fractions were prepared and the alkylating activity was assayed as described in the section on Methods. Values were determined with the aid of a continuous, simple, linear-regression program on an Olivetti Underwood Programma 101 (r = 0.98 for isophosphamide; r = 0.92 for cyclophosphamide).

TABLE 2.—Effect of pretreatment with phenobarbital, isophosphamide, and phenobarbital plus isophosphamide on microsomal metabolism*

Pretreatment	Activation of isophosphamide (μmol mechlorethamine equivalent/g of liver/hr)	Increase (%)	Activation of cyclophosphamide (μmol mechlorethamine equivalent/g of liver/hr)	Increase (%)	Ethylmorphine demethylase (μmol/g of liver/hr)	Increase (%)	Aminopyrine demethylase (μmol/g of liver/hr)	Increase (%)	Aniline hydroxylase (μmol/g of liver/hr)	Increase (%)
Saline	1.27 ± 0.20	—	2.11 ± 0.35	—	2.09 ± 0.18	—	2.57 ± 0.11	—	0.33 ± 0.06	—
Phenobarbital	3.48 ± 0.09	174	3.72 ± 0.31	76	4.58 ± 0.43	119	5.90 ± 0.65	130	0.96 ± 0.09	191
Phenobarbital plus isophosphamide	3.26 ± 0.61	157	3.12 ± 1.48	48	4.52 ± 0.48	116	5.89 ± 0.89	129	0.94 ± 0.02	185
Isophosphamide	1.60 ± 0.06	26	2.68 ± 0.36	27	2.74 ± 0.20	31	2.28 ± 0.43	−11	0.38 ± 0.01	15

*Animals received ip injections of isophosphamide (44 mg/kg of body weight), phenobarbital (55 mg/kg), or both daily for 8 days. Assays were carried out as described in the section on Methods. Each value represents the mean ± SE of three experiments (three animals in each group in each experiment).

TABLE 3.—The effect of pretreatment with phenobarbital and isophosphamide on liver microsomal protein and RNA and nuclear DNA*

Pretreatment	Microsomal protein (mg/g)	Microsomal RNA (mg/g)	Nuclear DNA (mg/g)
Saline	3.70 ± 0.44	0.37 ± 0.10	0.27 ± 0.01
Phenobarbital	5.78 ± 0.45	0.38 ± 0.07	0.29 ± 0.03
Phenobarbital plus isophosphamide	4.45 ± 0.73	0.35 ± 0.07	0.26 ± 0.02
Isophosphamide	3.84 ± 0.28	0.40 ± 0.01	0.26 ± 0.01

*Animals received ip injections of isophosphamide (44 mg/kg), phenobarbital (55 mg/kg), or both (in the same dose) daily for 3 days. Assays were carried out as described in the section on Methods. Each value represents the mean ± SE of three experiments (three animals in each group in each experiment). Values are given in milligrams per gram of wet weight of the liver.

mechlorethamine equivalents/g of liver/hour), but markedly different from the value reported by Cohen and Jao (0.5 μmol) (3). Our value for the Km of cyclophosphamide (4.0 millimols) lies between that of Sladek (1.4 millimols) and that of Cohen and Jao (7.0 millimols). The reason for the wide range of values obtained is not clear. Sladek found that freshly prepared microsomal fractions were required for the maximal activation of cyclophosphamide. We have confirmed this and have found that the microsomal activation of isophosphamide is also sensitive to the method of microsome preparation and storage. Other possible reasons for the differences between his own results and those of Cohen and Jao are discussed by Sladek. These include differences in the strain of rat used and the presence or absence of nicotinamide in the incubation mixture (6).

Recent reports indicate that species and sex also markedly affect the Vmax and Km values found (7,23).

The apparent Km for isophosphamide (significantly greater than that for cyclophosphamide) may be due to the presence of a chloroethyl group on the endocyclic nitrogen. It has been claimed that the metabolism of cyclophosphamide proceeds via the oxidation of the carbon atom adjacent to the endocyclic nitrogen (20–23). The bulky chloroethyl group on this nitrogen of isophosphamide may prevent close association of this substrate with a binding site on the microsomal enzyme surface. It should be pointed out, however, that since alkylating activity rather than substrate disappearance was measured in this study, and since equivalent amounts of isophosphamide and cyclophosphamide could give rise to a somewhat different number of mechlorethamine equivalents in the NBP reaction, it is possible that the difference in Km observed is not due entirely to differences in substrate affinity for the enzyme.

Although the induction of isophosphamide activation by phenobarbital is greater than that of cyclophosphamide (the difference being accounted for by the lower control value for isophosphamide), after induction the values for the two compounds are in the same range. This phenomenon (ie, the abolition of a pre-induction difference in the rate of microsomal metabolism of substrates which, chemically, are closely related) has been noted previously with hydrocarbon inducers but not with phenobarbital (24,25).

Tardiff and Dubois (26) found that pretreatment of rats with a single injection of cyclophosphamide (150 mg/kg administered ip) led to inhibition of microsomal *N*-demethylating activity. Twenty-four hours after treatment this activity was 66% of the control, and 5 days after treatment it was 20% of the control. Cyclophosphamide slightly decreased the level of microsomal enzyme induction produced by the administration of phenobarbital. In the present study, isophosphamide was administered in three daily doses (total dose, 132 mg/kg). No effect on microsomal demethylation or activation of isophosphamide or cyclophosphamide was observed, although there was a partial inhibition of phenobarbital-induced increases in microsomal protein. The difference may be due to the difference in the two drugs and to the fact that in the present study the dose was divided into three daily doses (7).

The phenobarbital-induced increase in isophosphamide activation was reflected in increased isophosphamide toxicity in the present investigation. Hemorrhagic cystitis, as indicated by frank hematuria, was seen in 12 of 12 animals receiving the combination but in none of 12 animals receiving isophosphamide alone.

The higher Km and lower rate of metabolism of isophosphamide (compared with cyclophosphamide at equal substrate concentration) corresponds with the higher tolerances to isophosphamide noted in clinical studies (10). The results of our study with phenobarbital suggest that pretreatment with phenobarbital may result in a greater increment in in vivo isophosphamide activity than in cyclophosphamide activity. Some of these results have been reported in a preliminary form (27,28).

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Pharmacologic Studies in Man With Crystallized L-Asparaginase (NSC-109229) [1,2,3]

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SUMMARY

Unlike earlier preparations of *Escherichia coli* L-asparaginase, the L-asparaginase currently available from Merck Sharp & Dohme has undergone a crystallization step during preparation. In order to determine whether this change has modified the pharmacologic characteristics of this enzyme, we have measured the levels of L-asparaginase and L-asparagine in the plasma of four patients with nonleukemic neoplasms who were treated intravenously with single doses of the crystallized enzyme (1500 international units/kg of body weight). The disappearance of L-asparaginase from the plasma was at least biphasic, with a rapid initial phase (half-time [$T_{1/2}$] of 4–9 hours) and a prolonged second phase ($T_{1/2}$ of 1.4–1.8 days). The concentration of L-asparagine was unmeasurable for 13–25 days. In view of the similarity between these results and results obtained previously in a similar study of four patients given noncrystallized L-asparaginase, we conclude that the addition of a crystallization step has not changed either the rate of disappearance of the enzyme from the plasma or the amidohydrolytic characteristics of the enzyme.

[Cancer Chemother Rep 56:611–614, 1972]

Escherichia coli L-asparaginase is an enzyme which is useful in the therapy for acute lymphocytic leukemia (1,2). L-Asparaginase preparations which disappear rapidly from the plasma lack antineoplastic activity, whereas preparations which disappear slowly may have an antileukemic effect (1–4). The rate of disappearance from the plasma varies markedly with the biologic source of the enzyme and can be modified further by physicochemical techniques (5). The *E. coli* L-asparaginase prepared by Merck Sharp & Dohme has been used in many pharmacologic studies (6–9). However, no data from studies comparing the currently available enzyme, which has been crystallized, with earlier, noncrystallized preparations, have been published. In order to determine whether crystallization has modified the pharmacologic properties of L-asparaginase, we have measured concentrations of L-asparaginase and L-asparagine in the plasma of four patients with nonleukemic neoplasms who were given single, large doses of the crystallized enzyme. The results obtained in this study indicate that the rate of clearance of L-asparaginase and the amidohydrolysis which occurred after the administration of crystallized L-asparaginase were indistinguishable

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[3] L-Asparaginase was supplied by the Cancer Therapy Evaluation Branch, DCT, NCI, through the courtesy of Drs. Max Tischler and Robert Denkewalter, Merck Sharp & Dohme.

[4] Solid Tumor Service, Medicine Branch, Clinical Oncology (Drs. Haskell and Canellos), and Laboratory of Toxicology, Experimental Therapeutics (Dr. Cooney), NCI.

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[7] The authors are indebted to Mrs. Victor Cohen and Mrs. Eleanor Weinstein for their technical assistance.

from the rate of clearance of the enzyme and the amidohydrolysis which occurred after the administration of a noncrystallized preparation of the enzyme (6).

MATERIALS AND METHODS

Four consenting adult patients received single doses of *E. coli* (EC-2) L-asparaginase which had been crystallized during preparation. The enzyme was administered intravenously (iv) over a 30-minute period at a dose of 1500 international units (IU)/kg of body weight. All patients had solid tumors not amenable to surgical treatment and resistant to standard therapy. Patient No. 1 had lymphosarcoma, patient No. 2 had carcinoma of the colon, and patients Nos. 3 and 4 had melanoma. These patients had normal liver and kidney function prior to treatment as determined by urinalysis and measurement of blood urea nitrogen, total serum bilirubin, serum alkaline phosphatase, and serum glutamic oxaloacetic transaminase levels. The patients did not experience either major toxicity or therapeutic response as judged by criteria described in detail previously (10).

Levels of L-asparaginase and L-asparagine in the plasma were determined by methods based on an enzymatically coupled oxidation of reduced pyridine nucleotide, as described in detail previously (6,11). The results were analyzed in the same way as in our earlier study with noncrystallized L-asparaginase (6). Specifically, values for the half-time of disappearance from the plasma ($T_{1/2}$) were established in both studies by plotting the data on semilogarithmic paper, drawing a line through the most linear segment of data points, and extrapolating this line back to time zero. The presence or absence of a statistical difference between the $T_{1/2}$ data of these two studies was evaluated by the Wilcoxon rank test (12).

RESULTS

Concentrations of L-asparaginase in the plasma of the patients treated in this study decreased in a curvilinear fashion which was at least biphasic, with an initial period of rapid decline in L-asparaginase levels, followed by a much slower decline. As shown in figure 1, the $T_{1/2}$ in this early phase varied between 4 and 9 hours. This was in sharp contrast to the much more prolonged disappearance rates seen later (fig 2), when the $T_{1/2}$ varied between 1.4 and 1.8 days. These latter $T_{1/2}$ values were compared

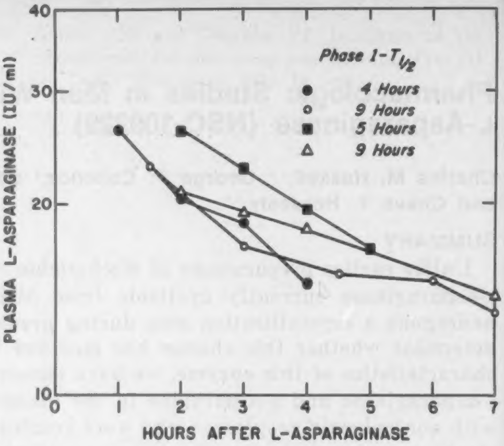

FIGURE 1.—Early phase of disappearance of L-asparaginase from plasma following an iv infusion of 1500 IU of the enzyme per kilogram of body weight at time zero hours. ●—● Represents patient No. 1; ○—○ represents patient No. 2; □—□ represents patient No. 3; △—△ represents patient No. 4.

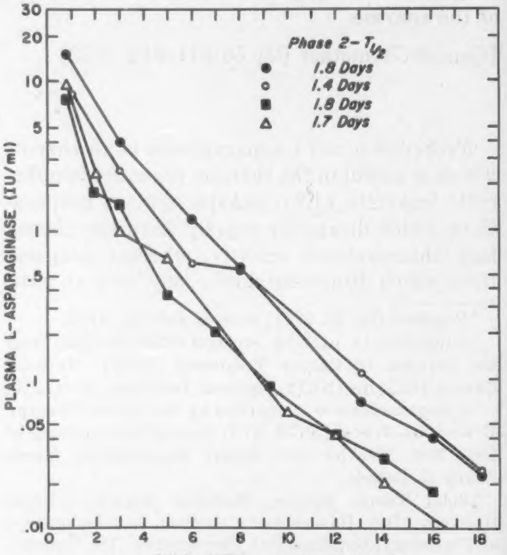

FIGURE 2.—Later phase of disappearance of L-asparaginase from plasma following an iv infusion of 1500 IU of the enzyme per kilogram of body weight at time zero days. ●—● Represents patient No. 1; ○—○ represents patient No. 2; □—□ represents patient No. 3; △—△ represents patient No. 4.

to those obtained by us in another study in which a noncrystallized preparation of L-asparaginase was used (6). A difference between the $T_{1/2}$ values of these two studies could not be demonstrated by statistical analysis ($P > 0.05$).

The effect of the single dose of L-asparaginase on the concentration of L-asparagine in the plasma of these patients was also assessed. In every case, plasma L-asparagine levels were unmeasurable for nearly 2 weeks after the administration of the large, single dose of L-asparaginase. Indeed, in two cases the duration of patient followup was inadequate for the assessment of the precise time of plasma L-asparagine reconstitution. Specifically, L-asparagine concentrations of 2.5 millimicromols/ml of plasma or less (the lower limit of sensitivity for the assay) were present for 13 days (patient No. 1), 17 days (patient No. 4), 14 or more days (patient No. 3), and 25 or more days (patient No. 2).

COMMENTS

L-Asparaginase can be obtained from many different organisms (including yeast, acid-fast bacilli, and many species of enteric bacteria) and from the plasma of many mammals (primarily members of the family Cavioidea and the New World monkeys) (1–3). Not all of these sources of enzyme yield therapeutically active forms of L-asparaginase. Two factors appear to correlate with these differences in efficacy: (*a*) differences in rates of clearance from plasma, and (*b*) differences in substrate specificity (3,4,13). Of these two factors, the former appears to be the more important. Broome has shown that L-asparaginase preparations which disappear rapidly from plasma fail to inhibit tumors implanted experimentally in animals (4). Riley and his co-workers extended these observations by demonstrating that infections with the lactic acid dehydrogenase (LDH)-elevating virus of mice may decrease markedly the rate of disappearance of L-asparaginase from the plasma of that animal, presumably by altering reticuloendothelial system clearance of the drug (13). Delayed disappearance in both cases appeared to correlate with an improved antineoplastic effect on sensitive forms of murine leukemia. As an extension of such studies, improvement in the therapeutic usefulness of *E. coli* L-asparaginase by chemical alteration of the L-asparaginase molecule has been attempted (5).

Because studies in animals have demonstrated a correlation between pharmacologic factors and therapeutic usefulness, and because the optimal method of preparing L-asparaginase may not yet be in use, one should confirm that changes in manufacturing techniques, such as the addition of a crystallization step during enzyme purification, have not altered the therapeutic efficacy of the drug. One approach to this control which is particularly applicable to L-asparaginase is to measure levels of the drug in the plasma and to determine the extent of L-asparagine amidohydrolysis by measuring L-asparagine concentrations in the plasma. Although the latter measurement poses some problems in interpretation because of the tenacity with which L-asparaginase resists denaturants (14), the concentrations of both L-asparaginase and L-asparagine were measured in this study for the purpose of comparison with similar measurements made in an earlier study of patients given a noncrystallized preparation of L-asparaginase (6). The similarity between the results of this and the earlier study suggest strongly that the addition of a crystallization step has not altered the pharmacologic properties of this commercial preparation of L-asparaginase.

A final, brief comment relates to our finding that the level of L-asparaginase in the plasma decreased in a curvilinear fashion which is nearly biphasic. Others have commented on this phenomenon as an occasional finding (9); however, similar studies with a variety of other enzymes suggest that a biphasic disappearance curve may be common for enzymes in man (15–18). It has been postulated that this reflects three processes (18): (*a*) a very rapid binding or trapping of some of the material at sites closely adjacent to the intravascular compartment; (*b*) a mixing phase (or phases) between intravascular and extravascular compartments lasting up to 6 days in man; and (*c*) while processes (*a*) and (*b*) are in progress, circulating and bound enzymes may undergo irreversible denaturation by exopeptidases or endopeptidases or by nonenzymatic mechanisms. Factors governing the relative importance of various removal mechanisms are unknown, but it is clear from the present study that conclusions regarding L-asparaginase clearance from plasma may be invalid if they are based on but short periods of observation.

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Further Clinical Trials With Porfiromycin (NSC-56410) (Large Intermittent Doses) [1,2,3]

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SUMMARY

One hundred and seventy-three patients were treated with porfiromycin in a phase I study of the drug. Data from 163 of the 173 trials were evaluable. The data suggest that intermittent treatment with 2.0-mg/kg doses of porfiromycin administered intravenously every 8 weeks as bolus injections can produce significant but manageable hematologic toxicity. Response to this schedule of intermittent treatment was observed in 32 of the 163 patients (19%) with evaluable trials. Included in the group of responding patients were five of 15 (33%) with carcinoma of the cervix, four of 14 (29%) with carcinoma of the ovary, three of 12 (25%) with carcinoma of the stomach, five of 27 (19%) with carcinoma of the head and neck, and seven of 28 (25%) with carcinoma of the colon. Other patients who responded had carcinoma of the thyroid gland, lung, or uterus, cloacal carcinoma, adrenal carcinoma, astrocytoma, or rhabdomyosarcoma. Further evaluation of this drug is indicated.

[Cancer Chemother Rep 56:615–624, 1972]

Porfiromycin ($C_{16}H_{20}N_4O_5$; molecular weight, 348.37) is a cytotoxic antibiotic obtained from *Streptomyces ardus* and *Streptomyces verticillatus.* It is the methyl derivative of mitomycin C, an antibiotic developed by Japanese investigators. The structure of porfiromycin is shown in figure 1. Cross resistance to porfiromycin in patients treated previously with other alkylating agents has not been reported; it did not occur in this clinical trial.

In an earlier study (1), we reported that porfiromycin can be useful in the treatment of disseminated cancer. Unusually long unmaintained responses were observed occasionally (*see* case report No. 1), making further evaluation of this drug worthwhile. Limiting toxic effects were leukopenia and thrombocytopenia which tended to be of long duration. Because of the tendency of hematologic toxicity to be cumulative, maintenance therapy was difficult.

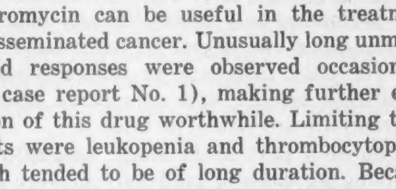

FIGURE 1.—Structure of porfiromycin.

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[1] Received Oct 27, 1971; revised June 5, 1972.

[2] Supported in part by grant CA-07177 and training grant T12 CA-08096 from the National Cancer Institute (NCI), National Institutes of Health (NIH), Public Health Service.

[3] Porfiromycin was supplied by Drug Research and Development, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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In the study reported here we attempted to explore further the usefulness of porfiromycin in the treatment of disseminated solid cancers and to find more practical dosage schedules by administering the drug infrequently at higher dose levels.

MATERIALS AND METHODS

Porfiromycin, a dark purple crystal, was supplied in lyophilized form. The contents of each 15-mg ampule were reconstituted with 10 ml of distilled water. One hundred and seventy-three trials were undertaken. Only those patients with diseases refractory to conventional chemotherapy, radiation therapy, or both, or diseases known to be unresponsive to conventional forms of chemotherapy were included in the study. The types of tumors treated are summarized in table 1.

White blood cell (wbc) and platelet counts were obtained three times weekly during the first 6 weeks and thereafter as indicated. Earlier in the study, liver function studies (measurement of serum alkaline phosphatase, serum glutamic oxaloacetic transaminase [SGOT], lactic dehydrogenase [LDGH], and bilirubin levels, and prothrombin time) were performed weekly. Because no nonhematologic toxic effects were observed in the first 150 patients treated (including those patients in the earlier reported study), the liver function studies were later performed only when clinically indicated. The incidence and degree of hematologic toxicity (ie, mild, moderate, or severe) observed in patients treated with increasing doses of porfiromycin are listed in table 2.

In an early part of the study, 1.0-, 2.0-, 2.5-, or 3.0-mg/kg doses of porfiromycin were given as intravenous (iv) bolus injections to 13 patients. The patients were then observed for 2–3 weeks or until recovery from drug-induced leukopenia and thrombocytopenia occurred (table 2). After the effects of a single large dose were known, an attempt was made to establish the interval required for the complete recovery of the bone marrow (as measured by the tolerance to subsequent doses of porfiromycin).

Porfiromycin was always administered as an iv bolus injection with precautions being taken to avoid paravenous infiltration.

TABLE 1.—Neoplasms treated with porfiromycin

Type or site of neoplasm		No. of cases
Large intestine		28
Head and neck		27
Nasal cavities, ear, accessory sinuses	12	
Nasopharynx	5	
Salivary gland	5	
Oropharynx	3	
Lip	2	
Cervix		15
Ovary, fallopian tube		14
Stomach		12
Rectum		10
Lung		10
Breast		6
Primary site unknown		6
Squamous cell carcinoma	3	
Anaplastic carcinoma	1	
Adenocarcinoma	2	
Testicles		6
Osteogenic sarcoma		5
Melanoma		4
Fibrosarcoma		4
Uterus		3
Astrocytoma		3
Pancreas		6
Kidney		2
Rhabdomyosarcoma		2
Leiomyosarcoma		2
Miscellaneous*		8

*This category included one case of each of the following: chondrosarcoma, Ewing's sarcoma, Hodgkin's disease, squamous cell carcinoma of the skin, esophagus, biliary, larynx, or mouth, carcinoma of the thyroid gland, and basaloid cell carcinoma of the rectum.

The various dosage schedules used are summarized in table 3. When the first injection had no toxic effect or only minimal toxic effects, patients were given identical amounts of the drug at intervals specified in table 3 in order to assess the increased bone marrow sensitivity. If tumor progression was observed, treatment was discontinued. Ultimately, a dosage schedule of 2.0 mg/kg every 8 weeks was established. For some patients, a decrease in dose or an increase in the interval between

TABLE 2.—Hematologic toxicity with increasing doses of porfiromycin*

Dose (mg/kg)	No. of patients treated	No. of patients with hematologic toxic effects		
		Mild	Moderate	Severe
1.0	4	—	—	—
2.0	4	—	—	—
2.5	2	—	1	—
3.0	3	—	2	—

*The degree of toxicity was defined as follows: mild toxicity = a wbc of 3000–4000 cells/mm³, a platelet count of 75,000–100,000 platelets/mm³, or both; moderate toxicity = a wbc of 2000–3000 cells/mm³, a platelet count of 50,000–75,000 platelets/mm³, or both; and severe toxicity = a wbc of less than 2000 cells/mm³, a platelet count of less than 50,000 platelets/mm³, or both.

treatments was necessary as toxicity occurred after the first, second, or third dose.

RESULTS

One hundred and seventy-three patients entered the study. Two of these patients died of advancing disease before toxicity could be evaluated. Eight others were excluded because sufficient data were not available for analysis.

A single bolus injection of porfiromycin was administered to 79 patients in the study. The treatment was not repeated if there was clinical evidence of disease progression or toxicity,[6] or if death occurred. Patients who responded to porfiromycin or had no evidence of progression of disease were given additional doses of this drug. The toxic effects observed in this group of patients are shown in table 3. Most of the patients who had toxic reactions to one injection of porfiromycin had previously received extensive radiation therapy or chemotherapy or both. Severe hematologic toxicity occurred after one dose of porfiromycin in 45% of the patients who had had three or more previous courses of treatment; however, no toxicity occurred after one dose in patients who had not received previous radiation therapy or chemotherapy.

Two doses of porfiromycin were given to 60 patients at the doses and intervals shown in table 4. After the second dose, the incidence of toxicity was significantly increased. The data suggest that the latent effects of the 2.0-mg/kg dose of porfiromycin had not entirely subsided after as long as 8 weeks.

Three doses of porfiromycin (starting dose, 2.0 mg/kg) were given to 19 patients (table 5). Four patients tolerated the dosage schedule with no toxicity. All of these four patients were considered "good risks." An additional five patients received treatment (2.0 mg/kg) every 8 weeks with moderate or severe toxicity occurring after the third dose. The other patients received reduced doses because toxicity occurred.

Six patients received either four, five, or six doses of porfiromycin. These patients required dosage adjustments (progressive reductions in dose and increased intervals between treatments) similar to those shown in figure 2 for a patient who received five doses of porfiromycin.

Toxic Effects

Hematologic toxicity was noted in about 45% of the patients receiving one dose of porfiromycin and in almost all patients receiving two or more doses of the drug. Leukopenia without thrombocytopenia or a fall in the hemoglobin value occurred in 80% of the patients receiving the first dose. Thrombocytopenia occurred in approximately 20% of those patients receiving the initial dose of porfiromycin and was as severe as the leukopenia. The nadir of the leukopenia caused by the first dose occurred 7–14 days after the treatment was given; recovery occurred 21–28 days after treatment.

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[6] Extravasation of the drug occurred in one patient with malignant melanoma. The resulting slough and skin ulcer caused the patient to refuse further treatment with the drug.

TABLE 3.—Hematologic toxicity* produced by a single injection of porfiromycin

Dose (mg/kg)	No. of patients treated	No. of patients with hematologic toxic effects		
		Mild	Moderate	Severe
1.2	1	—	—	—
1.5	3	—	1	1
1.8	4	—	1	2
2.0	71	20	4	6
Total	79	20	6	9

**See* footnote for table 2 for definitions of degree of toxicity.

TABLE 4.—Increasing hematologic toxicity* produced by a second dose of porfiromycin

Dosage	No. of patients treated	No. of patients with hematologic toxic effects—							
		After dose No. 1				After dose No. 2			
		No toxicity	Mild	Moderate	Severe	No toxicity	Mild	Moderate	Severe
Every 3 wks									
1.0 mg/kg	3	3				1	1	1	
1.2 mg/kg	3	3					1	1	1
Every 4 wks									
1.5 mg/kg	7	4	1	2		1	1	1	4
1.8 mg/kg	5	3	2				1	1	3
Every 6 wks									
1.5 mg/kg	6	4	2				4		2
1.8 mg/kg	7	4	3			3	1	1	2
Every 8 wks									
2.0 mg/kg	29	25	2	2		18	6	2	3

**See* footnote for table 2 for definitions of degree of toxicity.

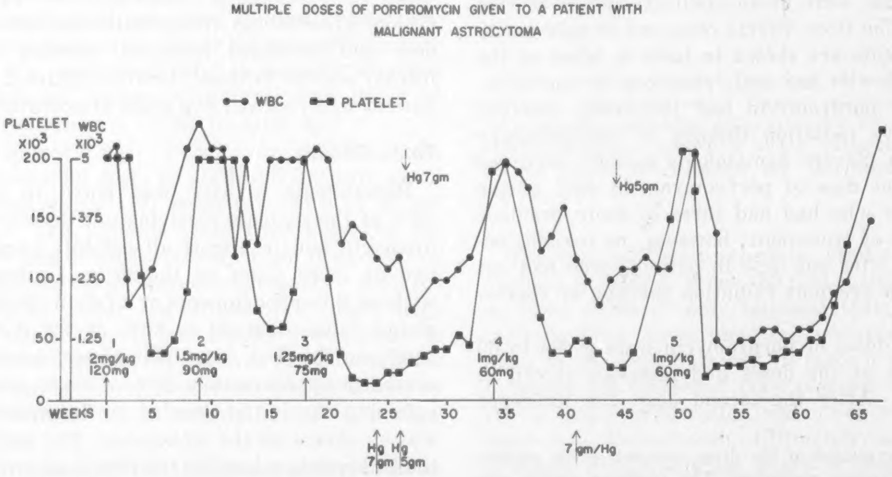

FIGURE 2.—Hematologic toxicity in a patient who received five doses of porfiromycin iv. The patient required intermittent blood and platelet transfusions after the 25th week of therapy.

TABLE 5.—Occurrence of hematologic toxicity* in 19 patients receiving three doses of porfiromycin at 8-week intervals

	First treatment		Second treatment		Third treatment	
	Dose (mg/kg)	Toxicity	Dose (mg/kg)	Toxicity	Dose (mg/kg)	Toxicity
1	2.0	None	2.0	Mild	2.0	Severe
2	2.0	None	2.0	Mild	1.5	Moderate
3	2.0	None	2.0	Mild	1.5	Mild
4	2.0	Mild	1.5	Mild	1.5	Mild
5	2.0	None	2.0	Mild	2.0	Moderate
6	2.0	None	2.0	Mild	2.0	Severe
7	2.0	None	2.0	None	2.0	Severe
8	2.0	Mild	1.5	Mild	1.5	Mild
9	2.0	None	2.0	Mild	1.5	Mild
10	2.0	Severe	1.0	Mild	1.0	Mild
11	2.0	None	2.0	Mild	2.0	Severe
12	2.0	Moderate	1.5	Mild	1.5	Mild
13	2.0	None	2.0	None	1.5	Moderate
14	2.0	None	2.0	Mild	1.0	Moderate
15	2.0	Mild	2.0	Severe	1.0	Moderate
16	2.0	None	2.0	None	2.0	None
17	2.0	None	2.0	None	2.0	None
18	2.0	None	2.0	None	2.0	None
19	2.0	None	2.0	None	2.0	None

**See* footnote for table 2 for definitions of degree of toxicity.

The onset and nadir of the thrombocytopenia occurred about 7 days after the onset of leukopenia and wbc recovery. With subsequent doses of the drug, wbc and platelet count nadirs often occurred in the first week after treatment and recovery was delayed as long as 15 weeks (*see* fig 2). The thrombocytopenia was as severe as the leukopenia in patients receiving more than one dose of porfiromycin.

Anemia was thought to be a consequence of porfiromycin toxicity in 30 patients. The anemia usually occurred after the second or third dose. The severity of the anemia paralleled that of the leukopenia and thrombocytopenia. Biphasic episodes of leukopenia or thrombocytopenia (ie, second depressions in wbc or platelet counts occurring several days after the apparent recovery of peripheral blood values) were observed frequently. Gastrointestinal disturbances (nausea) occurred in 12 patients. No stomatitis was observed. Several patients complained of a transient feeling of heat in the pelvic area shortly after they received the drug.

The urine of some patients became lavender blue for approximately 12–24 hours after the drug was administered, indicating excretion of the drug through the urinary tract. Several patients had purple bands on their nails after each dose of porfiromycin, indicating that the drug was incorporated into the nail with each treatment. Local extravasation of the drug caused severe pain and ulceration which required up to 1 year to heal and, in one patient, skin grafts. Hair loss occurred in 12 patients. In two patients, hair loss was complete.

Tumor Response

Objective tumor responses (greater than 50% reduction of all measurable lesions) were seen for a variety of tumors. Patients who did not have objective tumor responses after the first injection did not respond to subsequent doses. Fifteen patients with carcinoma of the cervix were treated. Five objective responses occurred, but response was noted only at sites not previously irradiated: the lungs, supraclavicular node, liver, and retroperitoneum. Recurrent pelvic masses in irradiated areas did not respond, regardless of the response of distant metastases. Patients with carcinoma of the cervix who responded to porfiromycin treatment tended to have longer disease-free intervals between the initial treatment and the detection of metastasis than did nonresponding patients (40 months vs 19 months, respectively). This prompts the observation that distant metastases were observed more often in patients whose primary tumors were locally controlled.

Fourteen patients with carcinoma of the ovary were treated with porfiromycin and four responded. All of the 14 patients had been treated previously and had been resistant to other forms of chemotherapy or had become resistant during treatment. Four of the patients who had previously responded to alkylating drugs responded to porfiromycin. No other patients with carcinoma of the ovary responded.

Carcinoma of the stomach was treated in 12 patients. Three patients responded. Adenocarcinoma of the large intestine or rectum was treated in 38 patients. Ten of the 38 had carcinoma of the rectum; 28 had carcinoma elsewhere in the colon. Four patients with cancer of the colon and three with cancer of the rectum responded to treatment. There was no correlation between these responses and previous responses to chemotherapy.

Carcinoma of the head and neck was treated in 27 patients. Five patients responded.

A summary of tumor responses is shown in table 6. Two additional cases are summarized in the next section. The report on the patient with a long unmaintained remission of carcinoma of the cervix has been reported previously (1). The other case (long remission of osteogenic sarcoma in a child treated with a dosage schedule reported in reference 1) has not been reported.

Case Reports

Case No. 1.—A 26-year-old married white woman with undifferentiated squamous cell carcinoma of the cervix was treated with radiation therapy in November 1964. One year later a metastatic nodule was removed from the left lung. In April 1966, a 2.5 × 2.5-cm nodule was excised from the right deltoid muscle. Examination of the nodule revealed metastatic squamous cell carcinoma.

The patient was seen by the Oncology Service on July 22, 1966. At that time, she complained of pain in the left hip. An axillary node on the right side was enlarged, measuring 6.0 × 3.5 cm, and there was a 7.0 × 5.5-cm mass in the left hamstring muscle. Histologic examination of this mass revealed squamous cell carcinoma. The results of metastatic bone survey, chest roentgenogram, and pelvic examination were negative. On July 24, 1966, weekly injections of porfiromycin (0.3 mg/kg) were begun. Although therapy had to be interrupted several times because of the occurrence of leukopenia and thrombocytopenia, it was continued until January 1968. At that time, there was no evidence of any metastatic disease and treatment was stopped. The patient remains free of disease and is employed in our hospital as a nurse's aide.

Case No. 2.—A 12-year-old girl was admitted to the hospital in October 1967 complaining of hemoptysis and pain in the anterior chest wall. Her symptoms had started 2 weeks prior to the time of admission.

Chest roentgenograms revealed mediastinal widening and a mass in the lower lobe of the left lung. Histologic examination of a biopsy specimen of the mediastinum revealed metastases from an osteogenic sarcoma. A bone survey revealed a nonsymptomatic destructive osteoblastic lesion in the upper end of the left tibia. After 5500 rads of cobalt 60 were delivered to the tibia (Co 60 teletherapy), the patient was referred to the Oncology Service.

Because the patient had mediastinal widening and palpable supraclavicular nodes, porfiromycin treatment was started. On December 18, 1967, a course of six weekly injections of porfiromycin (0.5 mg/kg) was begun. This treatment caused her wbc to drop to 2100 cells/mm^3 and the platelet count to 50,000 platelets/mm^3. The hematologic toxicity subsided in approximately 3 weeks. The patient has been receiving maintenance therapy (0.15 mg/kg administered iv every 2–3 weeks) to the time of this writing (August 3, 1971). Her supraclavicular nodes are not palpable. Chest roentgenograms reveal a calcified mass at the site of her original lesion in the lower lobe of the left lung. The mass is approximately 20% of its original size. Her bone lesion remains stationary. The patient is attending school and is functioning normally.

DISCUSSION

This study confirms previous observations that porfiromycin can be useful in the treatment of patients with disseminated cancer. Of particular interest is its activity in the treatment

TABLE 6.—Summary of responses to porfiromycin treatment

Patient No.	Diagnosis	Previous therapy, response	Objective evidence of response to porfiromycin	Duration of response (mos)	Interval between the first injection and response (days)	Dose at start of response (mg/kg)	Karnofsky rating*		Toxicity	Degree†
							At start of treatment	During response		
1	Adenocarcinoma, rectum	5-FUDR, yes	75% reduction of liver size	3	21	2.0	60	70	Leukopenia	Mild
2	Adenocarcinoma, rectum	None	Abdominal masses disappeared	10	28	2.0	90	100	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe
3	Adenocarcinoma, rectum	None	Relief of obstructive uropathy, disappearance of lung nodule	5	28	2.0	20	90	Leukopenia Thrombocytopenia	Mild Mild
4	Adenocarcinoma, colon	5-FU, no	Over 50% reduction in palpable abdominal mass	3	21	2.0	50	70	Leukopenia Thrombocytopenia	Moderate Moderate
5	Adenocarcinoma, colon	5-FU + vinblastine, no	Over 50% reduction in liver size and abdominal mass	3	21	1.5	40	90	Leukopenia Thrombocytopenia Anemia	Severe Severe Moderate
6	Adenocarcinoma, colon	5-FU, yes	50% reduction in liver and peritoneal mass	3	21	1.5	70	80	Leukopenia Thrombocytopenia	Mild Mild
7	Adenocarcinoma, colon	5-FU, yes	50% reduction of liver size	4	28	2.0	60	80	Leukopenia Thrombocytopenia	Mild Mild
8	Basaloid cell carcinoma, anus	Methotrexate, no	Disappearance of vaginal lesions	6	21	1.5	30	70	Leukopenia Thrombocytopenia	Severe Mild
9	Lymphoepithelioma, nasopharynx	Cyclophosphamide, no	Over 50% reduction in liver size	2	35	2.0	80	80	Leukopenia Thrombocytopenia Anemia Gastrointestinal	Severe Severe Mild Mild
10	Squamous cell carcinoma, tonsil	X-ray, yes; methotrexate, no	75% reduction of pulmonary metastasis	1	28	2.0	70	70	Leukopenia Thrombocytopenia	Mild Mild
11	Squamous cell carcinoma, head and neck	Radiation therapy, yes; methotrexate, no	50% reduction of cervical lymph nodes	2	28	2.0	60	70	Leukopenia Thrombocytopenia	Mild Mild
12	Squamous cell carcinoma, head and neck	Radiation therapy, yes	50% reduction in cervical adenopathy	4	21	2.0	50	80	Leukopenia	Mild
13	Squamous cell carcinoma, submandibular gland	Methotrexate, no	Disappearance of all evidence of disease (multiple pulmonary nodules and bilateral cervical lymphadenopathy)	12 (patient experienced myocardial infarction, no tumor found on autopsy)	35	2.0	40	60	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe

TABLE 6.—Summary of responses to porfiromycin treatment—Continued

Patient No.	Diagnosis	Previous therapy, response	Objective evidence of response to porfiromycin	Duration of response (mos)	Interval between the first injection and response (days)	Dose at start of response (mg/kg)	Karnofsky rating* At start of treatment	Karnofsky rating* During response	Toxicity	Degree†
14	Squamous cell carcinoma, poorly differentiated, cervix	X-ray, yes	75% reduction in liver size	5	21	2.0	60	80	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe
15	Squamous cell carcinoma, poorly differentiated, cervix	X-ray, yes	Disappearance of cervical lymph node; relief of obstructive uropathy	4	14	2.0	20	90	Leukopenia Thrombocytopenia Anemia Skin‡	Severe Severe Severe Mild
16	Squamous cell carcinoma, poorly differentiated, cervix	Cyclophosphamide, no	75% reduction in liver size	2	28	1.5	80	80	Leukopenia Thrombocytopenia Anemia Skin	Severe Severe Severe Mild
17	Squamous cell carcinoma, well differentiated, cervix	Radiation therapy, yes	Disappearance of lung metastases	5 (still in remission)	21	2.0	60	80	None	
18	Squamous cell carcinoma, undifferentiated, cervix	None	Disappearance of lung lesion	4 (still in remission)	28	2.0	60	80	None	
19	Papillary serous cystadenocarcinoma, ovary	ThioTEPA, no; chlorambucil, no	Disappearance of mass in right flank	6	35	2.0	60	80	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe
20	Papillary serous cystadenocarcinoma, ovary	Surgery, yes; cyclophosphamide, yes	50% reduction in abdominal masses; control of ascites	9	21	2.0	50	90	Leukopenia Thrombocytopenia	Severe Severe
21	Papillary serous cystadenocarcinoma, ovary	5-FU + cyclophosphamide, yes; NSC-51095, no	75% reduction in measurable mass in omentum	12	31	2.0	70	100	Leukopenia Thrombocytopenia Anemia	Mild Mild Severe
22	Teratoma, ovary	Surgery, yes; 5-FU + cyclophosphamide, no	75% reduction of abdominal masses	3	28	2.0	70	70	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe
23	Adenocarcinoma, stomach	None	75% reduction of abdominal masses	2	21	2.0	80	90	Leukopenia Thrombocytopenia	Mild Severe
24	Adenocarcinoma, stomach	5-FU, yes	75% reduction in rectal lesion; relief of obstructive uropathy	3	28	2.0	50	70	Leukopenia Thrombocytopenia	Mild Mild

25	Adenocarcinoma, stomach	None	Disappearance of rectal shelf	10	21	1.8	70	70	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe
26	Adenocarcinoma, uterus	X-ray, yes; progesterone, no; 5-FU, yes	75% regression of pulmonary nodules and pelvic mass	3	28	2.0	60	80	Leukopenia Thrombocytopenia Anemia	Mild Mild Severe
27	Leiomyosarcoma, uterus	None	Disappearance of pelvic mass	3	28	2.0	70	80	Leukopenia Thrombocytopenia Skin	Fatal Fatal Severe
28	Papillary follicular carcinoma, thyroid gland	Iodine 131, no; cobalt 60, no	75% reduction of sternal mass and tracheal obstruction due to enlargement of paratracheal node on right side	7	21	1.5	70	80	Leukopenia Thrombocytopenia Anemia	Severe Severe Severe
29	Astrocytoma, grade III	X-ray, yes	50% reduction in size of lesion; changes in brain scan; reversal papilledema	11	35	2.0	40	70	Leukopenia Thrombocytopenia	Severe Severe
30	Rhabdomyosarcoma, alveolar, head and neck	None	Disappearance of mass in flank	3	28	1.8	60	70	Leukopenia Thrombocytopenia	Mild Mild
31	Oat cell carcinoma, lung	Cyclophosphamide, yes	Disappearance of hepatomegaly	2	4	4.0§	40	70	Leukopenia Thrombocytopenia Anemia	Severe Severe Moderate
32	Adenocarcinoma, adrenal gland	Mitotane, no; 5-FU, no	Disappearance of hepatomegaly	9 (still in remission)	28	1.5	60	80	Leukopenia Thrombocytopenia	Mild Mild

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*See reference 3.

†*See* footnote for table 2 and text for definitions of degree of toxicity.

‡Skin toxicity = extravasation of drug with local discomfort.

§Patient received 2 mg/kg twice on same day by mistake.

of epidermoid carcinoma of the cervix, ears, nose, and throat, as well as cancer of the gastrointestinal tract and alkylating drug-resistant carcinoma of the ovary. Several patients who had carcinoma of the ovary which was resistant to alkylating drugs had good responses to porfiromycin. The drug can produce severe hematologic toxic effects. Patients in our study who failed to have clear evidence of tumor response during the 8-week interval before the second dose was administered did not respond after further therapy. In an earlier study (1), three porfiromycin dosage schedules were described: (*a*) 600 μg/kg weekly for 4–6 weeks; (*b*) 500 μg/kg twice weekly for 3–4 weeks; and (*c*) 200–250 μg/kg/day for 3 weeks. When these schedules were used, cumulative toxicity necessitating 8–10-week intervals between treatments for bone marrow recovery were not uncommon. A dosage schedule by which the drug could be injected safely at predictable intervals was sought.

Patients who have not received extensive previous chemotherapy and those who have had enough time to recover from previous chemotherapy can tolerate a 2-mg/kg dose of the drug well. Cumulative toxicity can be expected with repeated administration of the drug. Additional injections do not appear to be indicated if a patient fails to respond to the first injection of the drug. Generally, it appears to be safe to repeat the 2-mg/kg dose a second or third time after an 8- or 9-week interval if previous doses have produced no toxic effects or only minimal toxic effects.

Examination of bone marrow from patients in whom hematologic toxicity occurred revealed pancytopenia of all bone marrow elements. Approximately 30% of these patients received other drugs after they had received porfiromycin. Patients who had received three or more doses of porfiromycin tolerated other chemotherapy poorly.

Whether further prolongation of the interval between treatments could eliminate the cumulative toxicity remains to be seen. In any case, further exploration of porfiromycin in the treatment of cancer of the cervix, head, neck, and gastrointestinal tract, and refractory carcinoma of the ovary is indicated.

LIST OF COMPOUNDS

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-; Leukeran

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluoro-2′-deoxyuridine: NSC-27640; 5-FUDR

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Mitotane: NSC-38721: CAS reg. No. 53-19-0; ethane, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-; *o*,*p*′-DDD

NSC-51095: CAS reg. No. 13020-83-2; purine-6-yl-trimethyl ammonium chloride; TRAMP

Porfiromycin: NSC-56410; CAS reg. No. 801-52-5; methyl mitomycin C

Progesterone: NSC-9704; CAS reg. No. 57-83-0; pregn-4-ene-3,20-dione

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-; triethylenethiophosphoramide

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

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Effects of Combination Therapy With Bleomycin (NSC-125066 and Dibromodulcitol (NSC-104800) on Squamous Cell Carcinoma in Man [1,2,3]

Takao Ohnuma, James F. Holland, Kumao Sako, and Donald P. Shedd [4]

SUMMARY

Effects of the combination of bleomycin and dibromodulcitol were studied in 28 patients with squamous cell carcinoma of various organs and in one patient with basal cell carcinoma of the skin. Bleomycin was given at a dose of 15 mg/m^2 twice weekly, and dibromodulcitol was given at a dose of 3 mg/kg/day for 6 weeks. Toxic effects noted were alopecia, leukopenia and thrombocytopenia, hyperkeratosis, erythema, desquamation, pigmentation, and fever. In a small number of patients, vomiting, anorexia, weakness, weight loss, pain on micturition, oral ulcers, loss of nails, and venosclerosis at the injection site also occurred. In at least one patient, pulmonary infiltration was regarded as drug-induced. Dose-limiting leukopenia and thrombocytopenia were considered to be caused mainly by dibromodulcitol, and the other toxic reactions appeared to be caused by bleomycin. Five of 20 patients with squamous cell carcinoma of the head and neck and one of two patients with skin carcinoma responded with a greater than 50% reduction of tumor size lasting 1+ to 4 months. Those patients with well-differentiated squamous cell carcinoma had better response rates. This study confirmed that bleomycin was a drug active against squamous cell carcinoma and suggested that the addition of dibromodulcitol did not increase the response rate.

[Cancer Chemother Rep 56:625–633, 1972]

Bleomycin is an antineoplastic antibiotic complex obtained from fermentation products of *Streptomyces verticillus* (1). Initial clinical studies with bleomycin in Japan had shown antitumor activity in squamous cell carcinoma (2–7). Patients with sarcoma and malignant lymphoma also responded (3,6). Additional studies in Europe (8) and in the United States (9–14) confirmed these findings. In these studies, the spectrum of sensitive human tumors was extended to include testicular carcinoma. Responses were reported in a small number of patients with carcinoma of the breast (13), carcinoma of the ovary (8), carcinoma of the bladder (8,11), and malignant melanoma (3.6,8). The lack of pronounced bone marrow toxicity attributable to bleomycin is of particular interest; however, unusual skin and pulmonary toxic effects have been consistently described.

Dibromodulcitol is one of the α, ω-substituted

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[1] Received Mar 10, 1972; revised June 1, 1972.

[2] Supported in part by grant CA-05834 from the National Cancer Institute (NCI), National Institutes of Health, Public Health Service.

[3] Dibromodulcitol: CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-. Bleomycin was supplied by Bristol Laboratories, Syracuse, NY, through the Cancer Therapy Evaluation Branch (CTEB), Division of Cancer Treatment (DCT), NCI. Dibromodulcitol was supplied by the CTEB, DCT, NCI.

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hexitols with antitumor activity. In an initial clinical trial in Hungary, the drug was found to be effective against chronic myelocytic leukemia, Hodgkin's disease, and certain solid tumors, especially squamous cell carcinoma (15). Four of eight patients with upper respiratory tract carcinoma (two of laryngeal and two of tonsillar origin) had excellent therapeutic responses lasting more than 6 months. An additional three of eight patients showed subjective improvement. One patient with squamous cell carcinoma of six patients with bronchial carcinoma responded to the dibromodulcitol treatment. These responding patients with solid tumors received the drug at doses of 4–6 mg/kg of body weight daily until a white blood cell count (wbc) of less than 4000 cells/mm^3 resulted. The total dose given during the course of therapy varied from 3000 to 14,000 mg. In nine of 35 patients studied, bone marrow depression developed with leukopenia and thrombocytopenia.

A phase I study of dibromodulcitol in the United States showed less remarkable effects. Nevertheless, one of 13 patients with cancer of the head and neck and one patient with squamous cell carcinoma of the anus responded (16). Bone marrow depression developed in most of the patients treated at dose levels of 2.0 mg/kg and higher. In two patients who received the drug at a dose level of 3.5 mg/kg, massive gastrointestinal hemorrhage and death might have been drug-related.

These findings indicated that both bleomycin and dibromodulcitol were active against squamous cell carcinoma but caused toxic effects in different organs or systems. The promising antitumor effects of both drugs and the low incidence of bone marrow toxic effects of bleomycin prompted us to study whether the combination of these two drugs could produce an improved therapeutic effect.

MATERIALS AND METHODS

Bleomycin was mixed with normal saline solution and given twice weekly for 6 weeks either as a direct intravenous (iv) injection or via a running infusion at a dose of 15 mg/m^2 of body surface area. The drug was supplied in 15-mg (potency) ampules. The bleomycin dose level was chosen from our initial clinical study (14). This dose level was well within the therapeutic dose range and since most patients tolerated the drug for 6 weeks we were able to give the maximum cumulative dose.

Dibromodulcitol was given orally in a dose of 3 mg/kg/day for a total of 6 weeks. The development of leukopenia (wbc of less than 3000 cells/mm^3) or thrombocytopenia (platelet count of less than 75,000 platelets/mm^3) or both caused interruption of the treatment until the values returned to normal levels.

Twenty-eight patients with histologic diagnoses of squamous cell carcinoma of various sites and one patient with basal cell carcinoma of the skin were included in the study. There were 20 men and nine women. Their ages ranged from 40 to 79 years with a median of 55 years. All but two patients had had surgical treatment, radiation therapy, chemotherapy, or a combination of these treatments. All 29 patients had had recurrent or persistent disease. Those who had received previous treatment were entered into the study after reversible toxic effects had subsided. All of the patients had advanced neoplasms which were not amenable to conventional therapy, but they were expected to live more than 2 months. Patients with grossly abnormal liver function tests, blood urea nitrogen (BUN) test results more than 25 mg/100 ml, or compromised pulmonary function not related to their tumors were excluded from the study. All patients had blood cell counts within normal limits at the beginning of the study. Patients were entered into the study by consecutive nonrandom assignment. Informed written consent was obtained from the patient before beginning the study.

After 6 weeks of treatment patients were observed for an additional 2 weeks while not receiving chemotherapy to determine therapeutic effects and to await any delayed toxic effects. In a few patients who had either responded to the treatment or whose diseases remained static, a further course of the treatment was given. Response was classified as (*a*) complete (complete disappearance of palpable or measurable mass); (*b*) partial (a 50%–99% reduction in the area of a measurable mass or a greater than 50% reduction in one estimated diameter of a palpable but not measurable mass, in the absence of new lesion appearance or tumor progression elsewhere); (*c*) improvement (a 25%–49% reduction in the area of a measurable mass); (*d*) no change (a ±25% change in the initial tumor area); and (*e*) progressive disease (unambiguous increase in the tumor area of more than 25%).

For the classification of differentiation of head and neck squamous cell carcinoma, we used the classifications which appeared in the pathology reports of tissues examined in this Institute. Except in two instances, all histologic classification was based on biopsy of the recurrent tumor.

RESULTS

Of 29 patients entered into the study, 12 completed the planned schedule of treatment with both bleomycin and dibromodulcitol. Bleomycin treatment was interrupted in 13 patients: prohibitive toxic reactions of the skin occurred in four patients, leukopenia progressed after the interruption of dibromodulcitol treatment in four, pneumonia or pulmonary infiltration occurred in three, severe vomiting occurred in one, and clinical deterioration occurred in one. In two patients with toxic reactions of the skin and in one patient with leukopenia, bleomycin was resumed after the patients improved and the course of 12 injections was completed.

Dibromodulcitol therapy was interrupted in 20 patients for the following reasons: wbc counts fell to less than 3000 cells/mm^3 (with or without thrombocytopenia) in 16 patients; pneumonia or pulmonary infiltration occurred in three patients; and one patient with a toxic reaction of the skin failed to return to the clinic for treatment. The median duration of initial dibromodulcitol administration was 37 days. In two patients the treatment was resumed and completed (a total of 6 weeks of drug administration) after the improvement of their leukopenic condition. In an additional two patients, leukopenia developed in the last days of treatment, but the administration of dibromodulcitol was completed as scheduled.

Two patients who responded to the treatment and another two whose diseases were static at least 2 weeks after the first course of treatment had ended, received an additional drug as a second course of treatment. Three patients (one with a partial response and two with static disease) received the identical schedule of drugs in the second course of treatment, but due to progression of the disease or prohibitive toxic reactions, treatment was interrupted. Another patient who had a partial response received 20 mg/m^2 of bleomycin for 5 consecutive days and was then unmaintained until relapse.

TOXIC EFFECTS

Drug-induced toxic effects are summarized in table 1. Nearly every patient had mucocutaneous toxic reactions. Erythematous lesions, with or without subsequent desquamation and pigmentation, developed 4–58 days after the treatment (median, 34 days). These lesions occurred most often on the elbows and hands. In a few patients, they were noted on the shoulder, chest wall, and flank. Bleomycin therapy was interrupted in three of 14 patients because of the severity of the toxic reactions. Hyperkeratosis occurred 8–62 days after the start of treatment (median, 35 days). It occurred in some patients as a small nodule along the fingers or palms of the hands, and in others it became a hard swelling of the entire hand with some subcutaneous edema. In one patient, diffuse, hyperkeratotic pigmented lesions developed at the vein site where bleomycin had been injected. Pigmentation occurred along the folds in the palms of the hands, along the scratch marks, at the area of hyperkeratosis, and at the site of previous erythematous lesions. Pigmentation was first observed 22–62 days after beginning treatment (median, 34 days). In addition, loss of hair was commonly observed. This occurred after 10–69 days of treatment (median, 29 days), but rarely progressed to complete alopecia. In one patient, nearly every fingernail and toenail came off soon after severe erythematous desquamatory skin changes. In many patients, these cutaneous changes were noted simultaneously in varying degrees at several sites.

Leukopenia, with or without thrombocytopenia, occurred in 18 patients. The nadir of the leukocyte count ranged from 150 to 3700 cells/mm^3 with a median of 2900 cells/mm^3. The median nadir leukocyte count of all of the patients studied was 3400 cells/mm^3 and the median duration to the nadir was 40 days after the start of treatment. Dibromodulcitol treatment was interrupted whenever a leukocytic count of less than 3000 cells/mm^3 was observed, except when leukopenia developed during the last days of treatment in two patients. Interruption of treatment with dibromodulcitol was, in many

TABLE 1.—Toxic effects of bleomycin and dibromodulcitol combination therapy in 29 patients with squamous cell carcinoma

Toxic effects	No. of patients	Occurrence rate (%)
Mucocutaneous reactions	28	97
Hair loss	23	79
Hyperkeratosis and skin thickening	16	53
Erythema and desquamation	14	48
Pigmentation	13	45
Micturition pain	3	10
Oral ulcer	1	3
Nail loss	1	3
Hematologic reactions	18	62
Leukopenia (< 4500 cells/mm³)	18	62
Thrombocytopenia (< 140,000 cells/mm³)	6	21
Fever	11	38
Vomiting	3	10
Pulmonary reactions	2	7
Miscellaneous*	8	28

*Miscellaneous includes anorexia, weakness, loss of weight, phlebosclerosis at the site of injection.

cases, followed by a prompt return to normal of the leukocyte count without stopping treatment with bleomycin. In one patient, however, dibromodulcitol was withheld after 19 days of treatment when the leukocyte count had fallen from 5500 to 2100 cells/mm³ and the thrombocyte count had fallen from 335,000 to 137,000 platelets/mm³. Leukopenia and thrombocytopenia progressed to near absolute, and marrow aplasia was noted. The patient received platelet transfusions but died on Day 27 with a clinical picture which was compatible with hemorrhage of the central nervous system. Since we have not noted any pronounced hematologic toxic effects from this dose level of bleomycin in patients with normal bone marrow function, dibromodulcitol was regarded as the major cause of this complication.

Pulmonary complications developed in two patients. Exertional dyspnea developed in one patient on Day 28 after receiving eight injections of bleomycin. A roentgenogram of the chest showed diffuse markings in both lung areas. There was no evidence of infection or congestive heart failure. He died 1 month later. In the other patient with squamous cell carcinoma of the lung, exertional dyspnea and cyanosis developed. Roentgenograms of the chest showed scattered, fine, nodular infiltrates in addition to previously known tumor infiltrates. Administration of bleomycin was interrupted but he died 2 weeks later.

In about 40% of the patients, transient fever, with or without chills, developed 4–10 hours after an injection of bleomycin. In addition, some patients experienced weakness and gastrointestinal disturbances such as nausea, vomiting, "gas pains," and anorexia. Also, a small number of patients experienced dizziness, weakness, and weight loss.

THERAPEUTIC EFFECTS

Responses of patients to the treatment are summarized in table 2. Five of 20 patients (25%) with squamous cell carcinoma of the head and neck, and one of two patients with carcinoma of the skin responded with a greater than 50% tumor regression. Some examples of responses are shown in figures 1–4. The detailed information on these responses is illustrated in table 3. Among the six responders, only two received additional treatment as maintenance therapy. Three patients were not maintained because of residual toxic effects and the patients' refusal of further treatment. One patient did not receive the drug as maintenance therapy because he failed to return to the clinic for further treatment. Partial responses lasted 1+ to 4 months. There was no correlation between the degree of toxicity and the extent of response.

The relationship between the degree of differentiation and response in 20 patients with squamous cell carcinoma of the head and neck is shown in table 4. All responding patients with head and neck carcinoma had well-differentiated carcinoma. The one responder with skin carcinoma had moderately differentiated squamous cell carcinoma. These results suggest that better responses occur in the patients with more differentiated squamous cell carcinoma.

DISCUSSION

The present studies reveal definite therapeutic effects in patients with squamous cell carcinoma after treatment with the combination of bleomycin and dibromodulcitol. The develop-

TABLE 2.—Responses of patients to combination therapy with bleomycin and dibromodulcitol

Disease and site	No. of patients	No. of patients with— Partial response	Improvement	No change and progressive disease
Squamous cell carcinoma	28	6	2	20
Head and neck*	20	5	1	14
Lung	3	0	0	3
Skin	2	1	0	1
Penis	1	0	1	0
Uterine cervix	1	0	0	1
Anus	1	0	0	1
Basal cell carcinoma	1	0	0	1
Totals	29	6	2	21

*Squamous cell carcinoma of head and neck includes: tongue, 6 patients; floor of mouth, 4; vocal cord, 2; uvula, 2; one each of nasal sinus, nasopharynx, palate, lower lip, hypopharynx, and pyriform sinus.

TABLE 3.—Partial responses of patients treated with the combination of bleomycin and dibromodulcitol

Patient No.	Age, sex	Squamous cell carcinoma of—	Dose to response: Bleomycin (mg/m²)	Dose to response: Dibromodulcitol (mg/kg)	No. of days to response	Extent of response (%)	Duration of response (mos)	Remarks
1	45, M	Tongue	120	63	32	75	2	Maintained with same regimen until recurrence
2	45, M	Floor of mouth	105	60	32	75	2	Unmaintained (failed to return for treatment)
3	56, M	Tongue	150	96	69	75	1+	Unmaintained (residual toxic effects) for 1 mo, then received CCNU*
4	52, M	Lower lip	120	99	34	90	3	Unmaintained (residual toxic effects)
5	48, F	Skin	90	54	25	90	2	Unmaintained (residual toxic effects)
6	63, M	Hypopharynx	180	132	48	75	4	Received additional bleomycin (20 mg/m²) for 5 days, then unmaintained

*CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

TABLE 4.—Relation of degree of differentiation and response in squamous cell carcinoma of head and neck

Degree of differentiation	No. of patients	No. of patients with— Partial response	Improvement
Well-differentiated	10	5	0
Moderately differentiated	4	0	0
Poorly differentiated	6	0	1
Totals	20	5	1

ment of cutaneous toxic reactions in nearly 100% of the patients and the interruption of treatment in four patients indicate that bleomycin was used at the maximum tolerated dose. The same appears to be true for treatment with dibromodulcitol, since leukopenia developed in more than half the patients, with one instance of fatal bone marrow depression. Various cutaneous toxic reactions expected from treatment with bleomycin did not appear to be exaggerated by the addition of dibromodulcitol. The median leukocyte count depression was 50% of the pretreatment level and the median duration to the nadir was 40 days. These data are similar to results reported in treatment with dibromodulcitol alone. Keyes et al (17) found that daily administration of 100 mg/m^2 of dibromodulcitol produced a depression of the leukocyte count to 65% of the pretreatment level and administration of 130 mg/m^2 produced a depression to 30% of the pretreatment level on about Day 40. Therefore, the addition

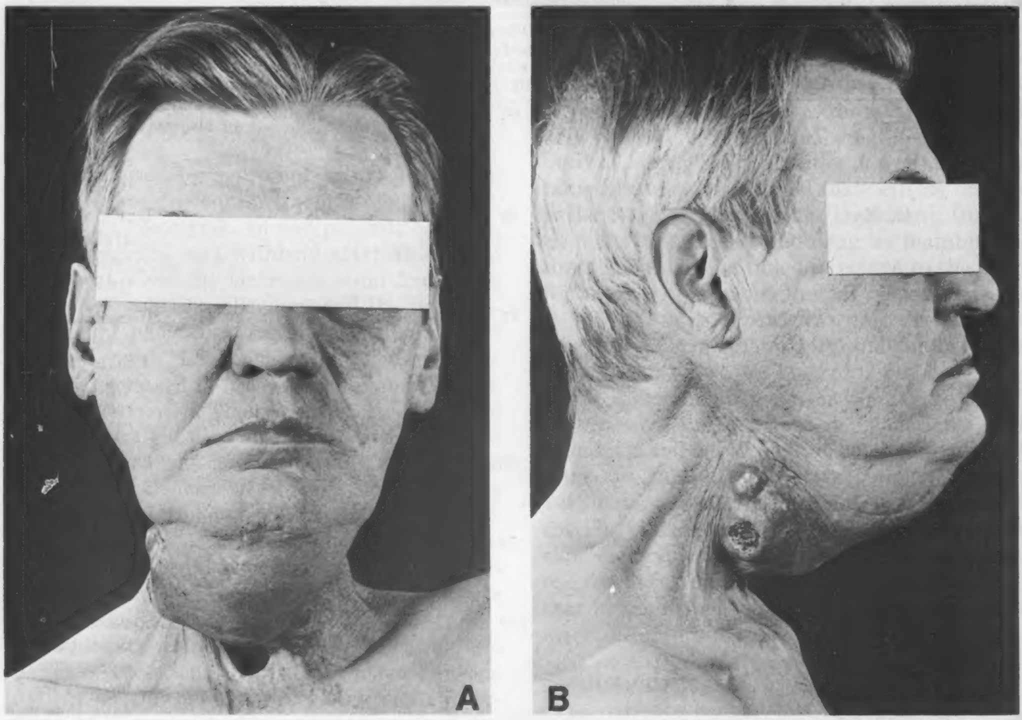

FIGURE 1 (A and B).—A 63-year-old man with well-differentiated squamous cell carcinoma of hypopharynx and a large neck metastasis, who had had radiation therapy, a pharyngolaryngectomy, and bilateral-radial neck dissections, prior to the treatment (Apr 14, 1971).

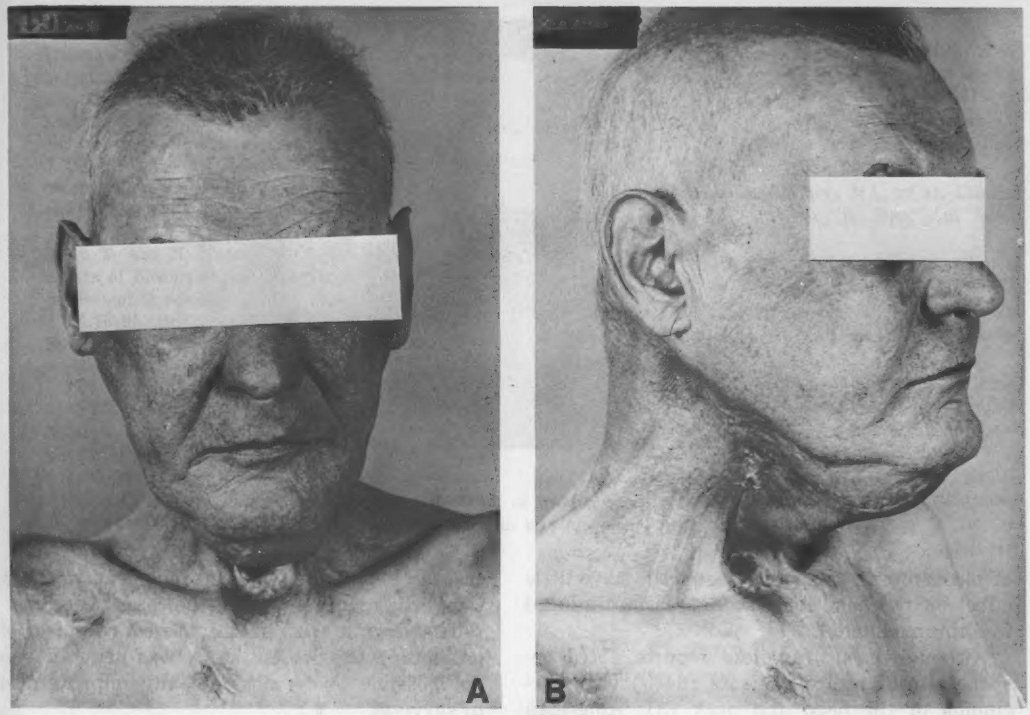

FIGURE 2 (A and B).—Same lesions shown in figure 1 after one course of treatment with bleomycin and dibromodulcitol (July 20, 1971). Note marked regression of neck tumor and hair loss.

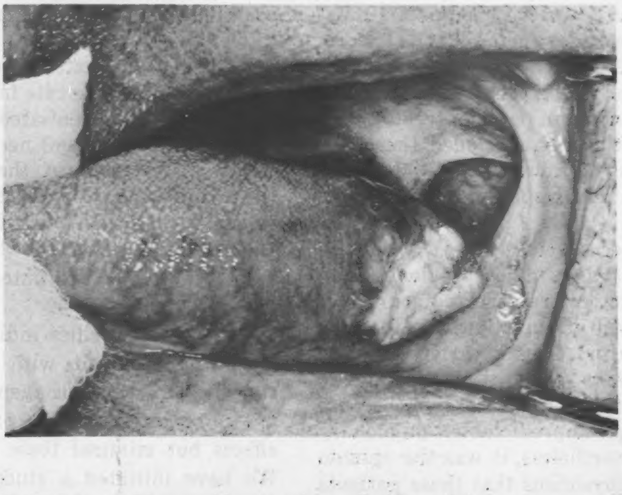

FIGURE 3.—A 58-year-old man with well-differentiated squamous cell carcinoma of the tongue prior to the treatment (Nov 13, 1970).

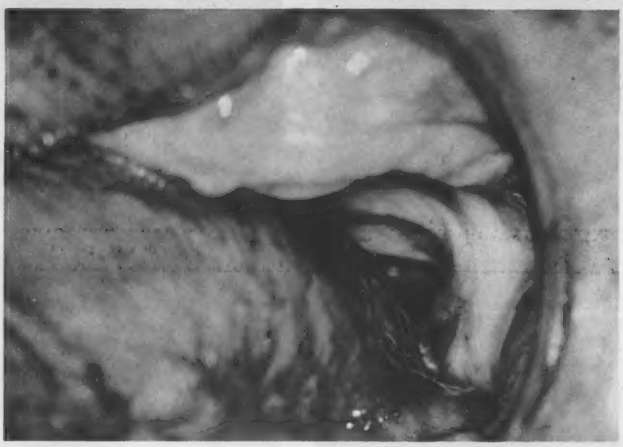

FIGURE 4.—Same lesion shown in figure 3 after a 6-week course of treatment with bleomycin and dibromodulcitol (Jan 22, 1971).

of bleomycin in this study seemed to have little effect on the bone marrow depression induced by dibromodulcitol.

Although early Japanese reports noted responses in 87 of 137 patients (64%) with carcinoma of the head and neck (3), American studies, reported in abstract form (9–12) and in a recent paper (13), noted responses in 11 of 47 patients (23%) with carcinoma of the same category. This is in accord with our own initial experience with bleomycin which showed partial response in four of 19 patients (21%) with head and neck carcinoma (14). The response of five of 20 patients (25%) with head and neck carcinoma in the present study is similar to the results noted in patients treated with bleomycin alone. It may be concluded, therefore, that the addition of dibromodulcitol did not increase the rate of response produced by bleomycin.

As has been noted previously (14), maintaining the responses was a major problem. Many patients advised to receive repeated courses of treatment expressed an unwillingness to do so because of discomfort from a variety of toxic reactions. Physicians were also reluctant to pursue intensive maintenance therapy because of late-developing, unpredictable, pulmonary complications. Nevertheless, it was the opinion of the attending physicians that those patients who were classified as having responded to the treatment benefited temporarily from a decrease in pain and an improvement in functional disturbance despite the toxic reactions.

The duration of unmaintained response of patients in the present study was brief; it was not possible to demonstrate any improvement in survival.

Prior investigators have made little comment of the influence of tumor differentiation in therapeutic responses. Since the present data are consistent with the previously noted effects of bleomycin alone, and do not indicate a unique contribution of dibromodulcitol, a prospective test of this finding can be made. Indeed, if the responsiveness is categorized by differentiation, the partial response rate in the present study is 50% for well-differentiated squamous cell carcinoma of the head and neck and 0 for ten patients with cancer of the head and neck of lesser differentiation. Such classification, if it is sustained, would significantly improve the value and indications for bleomycin in the treatment of well-differentiated carcinoma of the head and neck.

The present studies indicate that the combination of bleomycin with other myelosuppressive chemotherapeutic agents is practical. Other trials of drugs with promising antitumor effects but minimal toxic effects are possible. We have initiated a study of bleomycin and and adriamycin (NSC-123127) toward this end (18).

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Treatment of Ewing's Sarcoma With Adriamycin (NSC-123127)[1,2]

Robert K. Oldham and Thomas C. Pomeroy[3]

SUMMARY

Adriamycin was given to 11 patients with metastatic Ewing's sarcoma. They tolerated 60–75 mg/m² every 3 weeks without serious toxicity if therapy was not preceded by previous drug-induced pancytopenia. Prompt pain relief occurred in all 11 patients and objective responses (a greater than 50% reduction in measurable tumor mass) was noted in six of the 11 patients. Nausea, vomiting, and mild phlebitis occurred in all patients, while stomatitis and significant myelosuppression occurred only at the higher cumulative dose levels. Previous drug- or radiation-induced myelosuppression limited the dosage of adriamycin that could be administered. Adriamycin appears to be a useful chemotherapeutic adjunct in the treatment of Ewing's sarcoma.

[Cancer Chemother Rep 56:635–639, 1972]

Ewing's sarcoma is a malignant small cell tumor of bone with a propensity to widespread hematogenous dissemination. Previous studies from this department have documented the efficacy of combined radiation therapy and systemic chemotherapy in the primary treatment of this tumor (1,2).

Adriamycin is a hydroxymethyl analog of daunomycin[4] with demonstrated efficacy against acute leukemia and several solid tumors (3–6). Previous reports have indicated that both daunomycin and adriamycin can induce objective responses in patients with Ewing's sarcoma (4,5,7). In an effort to determine if adriamycin might be a useful adjunct in the treatment of Ewing's sarcoma, 11 patients with widely metastatic disease were treated and the results are summarized in this report.

MATERIAL AND METHODS

During 1971, 11 patients with widely metastatic Ewing's sarcoma were treated with adriamycin. The patients in this series were 7–25 years of age and included six males and five females. All but two were refractory to previous chemotherapy with cyclophosphamide, vincristine, and actinomycin D.[5] Four patients had also been previously treated with BCNU[6] with no objective response.

Although a variety of dosages were used initially, most of the patients were given 60–75 mg/m² of adriamycin intravenously over approximately 20 minutes. This infusion was repeated every 3–4 weeks depending on the patient's white blood cell count (wbc) and platelet count. Cases were evaluated before each treatment by clinical examination and appropriate

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[1] Received Mar 17, 1972; accepted June 13, 1972.

[2] Adriamycin was supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] Radiation Branch, Division of Cancer Biology and Diagnosis, NCI, Bethesda, Md.

[4] Daunomycin: NSC-82151; CAS reg. No. 23541-50-6.

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.
Vincristine: NSC-67574; leurocristine, sulfate (1:1).
Actinomycin D: NSC-3053.

[6] BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

TABLE 1.—Patients with Ewing's sarcoma treated with adriamycin

Patient No.	Drug dose	Concomitant therapy	Major toxic effects	Response	Followup*
1	60 mg/m^2, q3wks	None	Stomatitis	Pelvic mass regression >50%. Abrupt relief of bone pain with each dose.	Died 6 mos later with small pulmonary nodules. Possible cardiac toxicity.
2	60 mg/m^2, q3wks	None	None	Pulmonary nodules regressed 75% with first dose	Adriamycin followed by whole lung and mediastinal radiation therapy. NED 4 mos later.
3	60 mg/m^2, q3wks	None	Pancytopenia† (late)‡	Pulmonary nodules regressed 25%. Prompt relief of bone pain with each dose.	Stable with widespread metastasis at 6 mos
4	15 mg/m^2, twice per wk over 8 mos	None	None	Chest nodes regressed 50%	Recurrent primary tumor at 8 mos
5	25 mg/m^2/mo, later increased to 60 mg/m^2/mo	None	Pancytopenia,† mild stomatitis	Pulmonary mass regressed 50%. Right pleural effusion cleared. Prompt relief of bone pain.	Died 7 mos later with widely metastatic disease
6	60 mg/m^2, q3wks	Radiation therapy to left femur. None to right ischium.	Stomatitis, pancytopenia† (late)‡	Prompt relief of bone pain in all areas	Died 5 mos later with widespread metastasis
7	75 mg/m^2, q3wks	None	None	Complete disappearance of lung nodules	NED at 2 mos
8	60 mg/m^2, q2mos	Radiation therapy to primary mass	Fever with tumor regression	Very rapid and complete regression of 1° tumor and soft tissue mass	NED at 2 mos
9	25 mg/m^2/mo	Radiation therapy for localized bone pain just prior to adriamycin	Severe stomatitis. Severe pancytopenia.†	>75% regression of pulmonary nodules. Bilateral pleural effusion cleared. Prompt relief of bone pain.	Died with metastatic disease 3 mos later
10	60 mg/m^2, 1 dose	Radiation therapy	Mild pancytopenia	Prompt relief of bone pain before radiation therapy	Pelvic mass smaller at 2 mos
11	60 mg/m^2, q4wks	None	Mild pancytopenia	Complete relief of bone pain	NED at 1 mo
12	75 mg/m^2, q3wks	None	None	Supraclavicular node and lumbar mass regressed >50%. Prompt relief of bone pain.	NED at 3 mos. Alveolar rhabdomyosarcoma.

*NED = no evidence of disease.

†Previous BCNU-induced myleosuppression.

‡After high cumulative total dose.

roentgenograms. An additional 34-year-old man with metastatic alveolar rhabdomyosarcoma was treated in a similar manner and is included in the report.

RESULTS

The results are summarized in table 1. The most striking feature was the prompt relief of bone pain within hours in several of the patients and within 3 days in all of the patients. An objective response was defined as a greater than 50% reduction in a measurable lesion. Six of the 11 patients in this series had objective responses.

Two of the patients (Nos. 2 and 8) had not received previous chemotherapy and are in complete remission more than 4 months after beginning adriamycin. Figure 1 (patient No. 5) and figure 2 (patient No. 2) demonstrate the chest lesions prior to adriamycin. Figure 3 (patient No. 5) and figure 4 (patient No. 2) illustrate examples of objective responses to adriamycin.

Toxicity in this series was generally moderate with no drug-related deaths. Nausea, vomiting, phlebitis, and some alopecia were noted in all patients. Stomatitis and myelosuppression appeared to be dose-related and fairly predictable. Those patients with previous drug-induced myelosuppression exhibited the most severe pancytopenia, but in only three of the 11 patients were platelet transfusions required (two of whom had received recent BCNU therapy). Wbc counts dropped to less than 1000 cells/mm^3 in the four patients who had received prior BCNU therapy. The time-course of myelosuppression was also noted to be predictable with the nadir of wbc depression occurring at approximately 2 weeks and the nadir of platelet count depression occurring somewhat later, between 2 and 3 weeks after the start of therapy. In the absence of previous myelosuppression, both the wbc counts and platelet counts rebounded sharply toward normal. One patient (No. 3) had cardiac irritability (tachycardia and premature ventricular beats) during an episode of septic shock leading to death. At postmortem examination, mild fibrosis and cardiac dilatation were seen. This represents the only case with *possible* cardiac toxicity seen in this series.

DISCUSSION

Cyclophosphamide, vincristine, and actinomycin D have all demonstrated significant antitumor activity against Ewing's sarcoma and

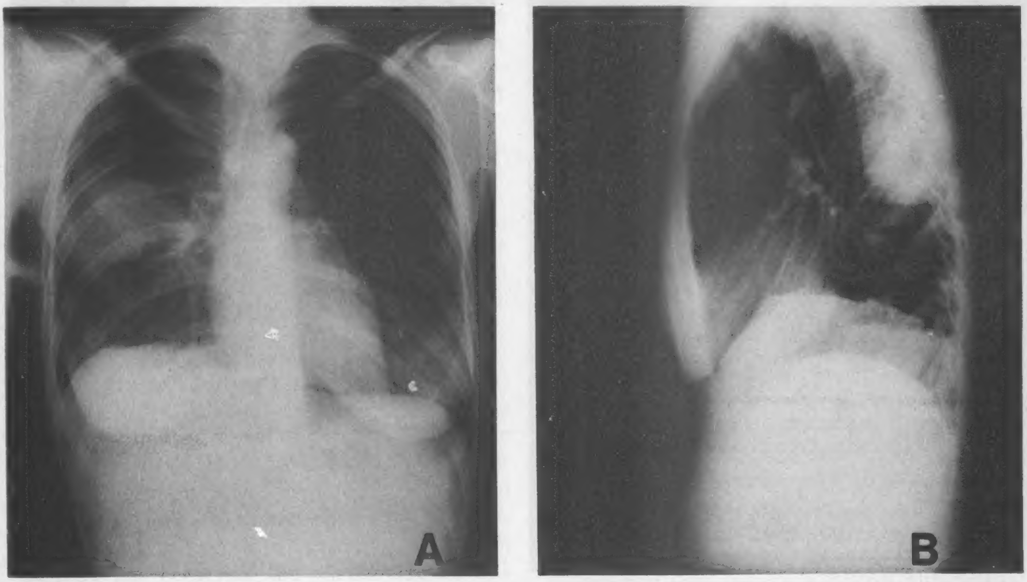

FIGURE 1 (*A and B*).—Chest lesions (patient No. 5) prior to adriamycin treatment.

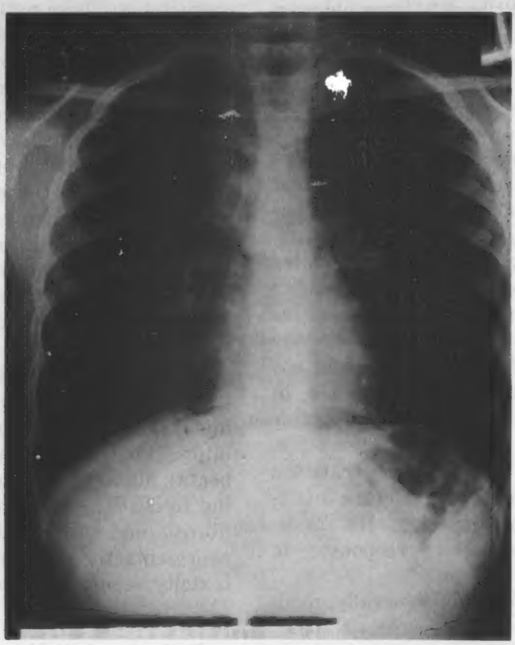

FIGURE 2.—Chest lesions (patient No. 2) prior to adriamycin treatment.

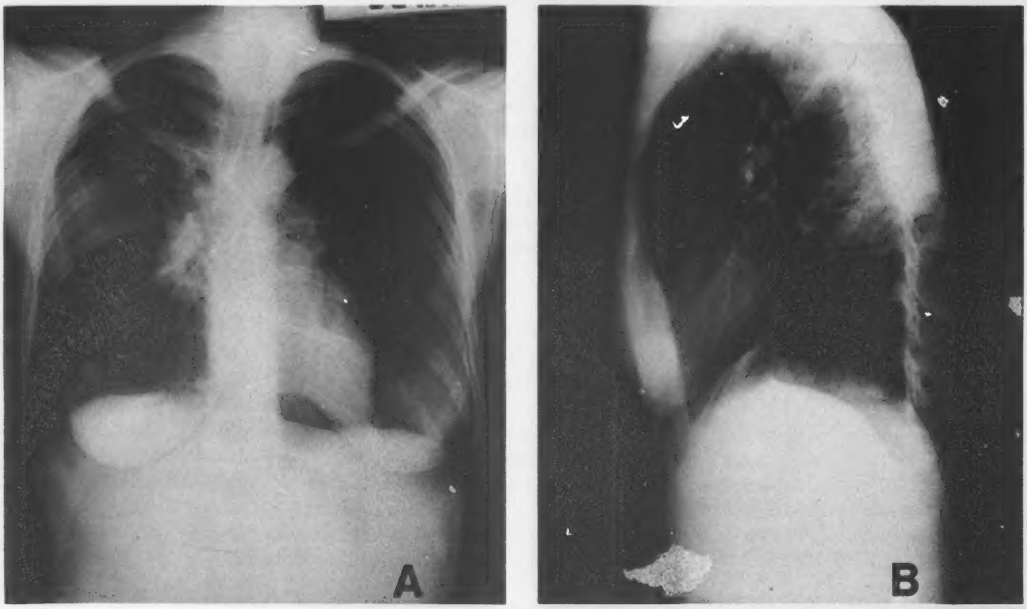

FIGURE 3 (*A and B*).—Objective response to adriamycin treatment (patient No. 5).

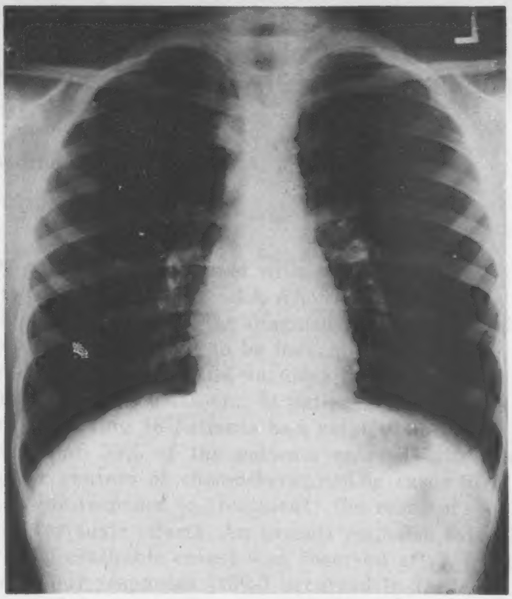

FIGURE 4.—Objective response to adriamycin treatment (patient No. 2).

are being used here as well as elsewhere for adjuvant chemotherapy (2). Our experience now indicates that adriamycin is useful in the treatment of Ewing's sarcoma even when the tumor is refractory to cyclophosphamide, vincristine, actinomycin D, and BCNU. Its prompt effect on bone pain as well as the high incidence of objective responses would indicate that adriamycin might be best utilized in the primary therapy of Ewing's sarcoma.

Of interest is the prolonged and severe myelosuppression which occurred in four of four patients in this series who had received BCNU (without objective response) prior to adriamycin. For combination chemotherapy to be useful, the individual agents must be effective and their additive toxicity must not preclude their use. Adriamycin appears to fit these criteria with two precautions: (*a*) when large marrow reserves have been irradiated, significant myelosuppression may follow adriamycin treatment; and (*b*) where previous drug-induced myelosuppression (particularly with BCNU) has occurred, the adriamycin at this dose level will regularly cause severe myelosuppression requiring supportive therapy. Adriamycin is now being evaluated in combination with cyclophosphamide and vincristine in the cyclic primary chemotherapy of patients with Ewing's sarcoma.

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[illegible]

are being seen here as well as elsewhere [illegible] chemotherapy [illegible]

[illegible]

Phase II Study of Carbestrol (NSC-19962) in Patients With Solid Tumors [1,2,3]

J. Kogler,[4] G. Hill,[4] N. Sedransk,[5] D. R. Cole,[6] A. J. Weiss,[7] and W. Wilson [8,9,10]

SUMMARY

In a phase II study, 96 patients were treated with 100 mg of carbestrol given orally twice a day for 6 weeks. All 96 patients had measurable lesions and histologic proof of the diagnosis of cancer. Each had metastatic disease which was believed to be incurable by the conventional means of radiation therapy and surgical treatment. Forty-four of the 96 patients had sarcomas and 34 patients had cancer of the breast. The remaining 18 patients had solid tumors of other various types. More than 50% of the patients entered into this study had one to four prior courses of chemotherapy. The cases of 80 patients were evaluated for response to treatment; the cases of 86 patients were evaluated for toxic effects. An overall response rate of 13.3% (11 responses out of 80 evaluable cases) was observed after treatment with carbestrol. Four responses (10%) occurred in the patients with sarcomas and three responses (10%) occurred in the patients with carcinoma of the breast. Side effects included gastrointestinal toxicity (26%), vaginal bleeding (9%), and edema (6%). Respiratory side effects and bone marrow depression were reported in 10% and 6% respectively, but could not be ascribed with certainty to carbestrol. Nephrotoxicity and liver toxicity did not occur, and no patients died of drug toxicity. This study showed that carbestrol was not a reliably effective cancer chemotherapeutic agent in the treatment of patients who had sarcomas and had received extensive prior chemotherapy. Therapeutic trials in patients with carcinomas of the breast who have not received any prior estrogen treatment may be fruitful.

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[2] Supported by research grant CA-06071 from the National Cancer Institute (NCI), National Institutes of Health, Public Health Service.

[3] Carbestrol was supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI.

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[7] Department of Surgery, Jefferson Medical College, Philadelphia, Pa.

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[9] This phase II study was conducted by the Central Clinical Drug Evaluation Program, *A. R. Curreri*, chairman, and *R. O. Johnson*, co-chairman. The following cooperating institutions and investigators contributed to the study: *Robert Ozeran*, Wadsworth VA Hospital, Los Angeles, Calif; *William Shingleton*, Duke University Medical Center, Durham, NC; *Stanley Altman*, Latter Day Saints Hospital, Salt Lake City, Utah; *Frederick Golomb*, Bellevue Medical Center, New York, NY; *George N. Cornell*, Cornell Medical Center, New York, NY; *Robert Madden*, New York Medical College, New York City; *William S. Fletcher*, University of Oregon Medical School, Portland; *Albert Segaloff*, Alton Ochsner Medical Foundation, New Orleans,

Carbestrol is a synthetic estrogen which has antitumor activity. It has a nonsteroidal structure (*see* fig 1) and its empirical formula is $C_{17}H_{22}O_3$.

Significant antitumor activity was observed in the following animal tumor systems: Sarcoma 180, mammary adenocarcinomas R35 and 13762, prostatic squamous cell Carcinoma 11095, and acute monocytic leukemia R3149.[11] The estrogenic effect was proved by vaginal cornification in rats who had had ovariectomy and by uterotrophic activity in castrated rats (The Upjohn Co., unpublished data, 1956). Carbestrol appeared to be a more potent estrogen in rats than either estradiol or diethylstilbestrol. In monkeys, however, it appeared to be considerably less potent than other standard estrogenic compounds. Rats treated with carbestrol on a 5 mg/kg/day dose schedule for 30 days showed a marked slowing of growth rate with weight loss during the first 10 days of treatment. At autopsy, the seminal vesicles, ventral prostate, testes, and thymus of the treated male, and the thymus of the treated female, had a smaller organ-body weight ratio than those of the control animals. Liver-, heart-, kidney-, and adrenal-body weight ratios were larger than in the control animals. Microscopic tissue examination showed atrophy of the seminiferous tubules and reduced spermatogenesis. There was thymic involution in both sexes. During recovery, pathologic changes in the organs became more marked.

Doubling the initial 5 mg/kg/day dose each week for 6 weeks caused additional slowing of the growth rate but did not influence mortality. More profound pathologic changes, such as ovarian atrophy and myometrium enlargement, were noted. After a 2-week recovery period, examination of organ tissues showed metaplasia of the uterine wall and the prostatic epithelium.

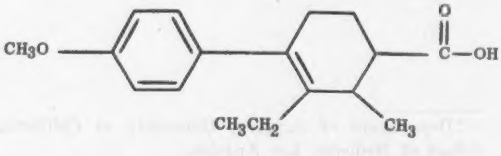

FIGURE 1.—Chemical structure of carbestrol.

In an unpublished phase I study carried out by Segaloff at the Alton Ochsner Medical Foundation, New Orleans, Louisiana, 18 patients were treated with carbestrol given orally in a dose range of 2.5–200 mg/day. Objective regression occurred in two patients, both of whom had sarcomas. Side effects observed during therapy were nipple pigmentation, ecchymosis, edema, nausea, and vomiting. All of these conditions were reversible.

The above mentioned data resulted in a phase II study, primarily of patients with carcinomas of the breast and sarcomas, using 100 mg of carbestrol twice a day for 6 weeks. An estrogenic compound with an additional antitumor activity was expected to be more effective than a single hormonal or cytotoxic compound in metastatic carcinoma of the breast. In addition, the encouraging results in Segaloff's phase I study warranted the evaluation of carbestrol in the treatment of patients with sarcomas.

CLINICAL DATA

Patients

There were 96 patients entered into this study between April 1969, and February 1971. None of these patients had had prior chemotherapy with carbestrol or analogs of carbestrol. No concomitant drug was given that might have affected their malignant tumors. Forty-four cases of sarcoma and 34 cases of carcinoma of the breast were investigated. The remaining 18 patients had other solid tumors of various types. Sixteen of these patients were not evaluable: four were excluded because of protocol violations concerning regular followup procedures, six died shortly after beginning therapy, and in the other six the drug had to be discontinued because of side effects.

Response

The responses are classified in accordance with the criteria used by Drug Research and Development, NCI: (*a*) complete response

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La; *Robert Edmunds*, St. Luke's Hospital Center, New York, NY; *Anthony Cruz*, University of Texas Medical School at San Antonio; *Guillermo Ramirez*, University of Wisconsin Medical Center, Madison.

[10] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[11] The unpublished data in this section were compiled and interpreted by C. V. Piczak, Endocrine Evaluation Branch, Division of Cancer Biology and Diagnosis, NCI.

(CR)—all signs and symptoms of tumor disappear; (*b*) partial response (PR)—at least a 50% decrease in the maximal cross-sectional tumor area, without simultaneous progression of the disease elsewhere; (*c*) no change (NC)—no progression or improvement in symptoms or signs of the tumor; (*d*) progression (P)—a measurable increase in tumor size, development of new lesions, or definite worsening of the patient's condition. Responses were observed in 13.3% (11 responses out of 80 evaluable cases) after treatment with carbestrol (table 1). Complete response was seen in two patients and partial response occurred in nine patients. In 46 patients the disease remained static and in the remaining 23 patients the disease progressed.

TABLE 1.—Overall response in 80 evaluable cases

Response category*	Response rate (%)
CR	2.5
PR	11.3
NC	57.5
P	28.7

*CR = complete response; PR = partial response; NC = no change; P = progression.

Drug-Related Toxicity

Gastrointestinal side effects, mainly nausea and vomiting, were experienced by 21 patients (table 2). In five cases toxicity necessitated the cessation of therapy; in one case the drug was withheld temporarily. Diarrhea, stomatitis, and anorexia of a slight degree occurred in three patients but did not cause any change in therapy. Respiratory side effects occurred in eight patients. Six of these patients had symptomatic metastatic spread to the lungs and the other two patients were entered into the study with pre-existing respiratory symptoms. No response to chemotherapy was shown by these eight patients. Whether the aggravation of these respiratory symptoms can be considered to be drug-related remains questionable. Vaginal bleeding was experienced by seven patients. Carbestrol was discontinued in two of these patients, and in three patients the drug was withheld for 14–28 days. Bone marrow depression was noted in five patients. Two of these patients had bone metastases with pre-existing signs of pancytopenia. Another two patients were entered into the study with white blood cell counts of 4.6 and 4.5 × 10^3 cells/mm^3, which dropped to 3.1 and 3.2 × 10^3 cells/mm^3. No significant change in the platelet count was observed. Edema developed in five patients during therapy. A skin rash was observed in one patient which subsided in spite of the continuation of carbestrol. Dizziness, the only central nervous system symptom, was felt to be drug-related in one case. Nipple pigmentation developed in two patients and one patient complained of fullness in her breast. Cystitis, stomatitis, and lymphangitis were encountered by these patients during therapy.

TABLE 2.—Side effects in 86 evaluable cases

Side effects	Occurrence rate (%)
Gastrointestinal tract	26.3
Nausea	
Vomiting	
Diarrhea	
Anorexia	
Respiratory system	10
Dyspnea	
Vaginal bleeding	8.7
Edema	6.3
Bone marrow depression	6.3
Infections	3.7
Cystitis	
Stomatitis	
Lymphangitis	
Breast	3.7
Nipple pigmentation	
Fullness	
No side effects	35

Results in Sarcomas

Forty-four patients with sarcomas were treated; the cases of 38 were evaluable. Only three of these patients had not had prior therapy. The age range of the 44 patients was 29–77 years. Of these patients, 72% had had prior surgical treatment for their primary tumors, 53% had received one to three courses of chemotherapy previously, and 37% had received radiation therapy. The histologic types and responses are listed in table 3. One complete and three partial responses were observed. The complete response occurred in a 28-year-old woman who had a fibrosarcoma of the greater trochanter. Her only prior treatment was internal fixation of a pathologic fracture. After 4 weeks of treatment the primary tumor was

TABLE 3.—Responses of patients with sarcomas to carbestrol

Disease	Evaluable	Not evaluable	Total	Response* CR	PR	NC	P
Liposarcoma	5	2	7	0	1	3	1
Leiomyosarcoma	10	1	11	0	0	6	4
Fibrosarcoma	3	0	3	1	0	0	2
Osteogenic sarcoma	4	1	5	0	0	2	2
Synovial cell sarcoma	5	0	5	0	0	3	2
Unclassified sarcoma	3	0	3	0	1	1	1
Anaplastic sarcoma	1	0	1	0	0	0	1
Rhabdomyosarcoma	2	0	2	0	0	1	1
Reticulum cell sarcoma	1	0	1	0	0	1	0
Lymphosarcoma	0	1	1	0	0	0	0
Chondrosarcoma	2	0	2	0	0	1	1
Spindle cell sarcoma	1	0	1	0	1	0	0
Ewing's sarcoma	1	0	1	0	0	1	0
Neurofibrosarcoma	0	1	1	0	0	0	0
Totals	38	6	44	1	3	19	15

*CR = complete remission; PR = partial remission; NC = no change; P = progression.

no longer measurable, and on the roentgenogram the defect was obliterated by new bone formation. This response persisted for 8 weeks. The three partial responses were noted in patients who had sarcoma of the colon, liposarcoma, and chondrosarcoma. The first patient had had no prior treatment and the second patient had received radiation therapy to his primary tumor. The patient with chondrosarcoma had received radiation therapy and consequently failed to respond to porfiromycin.

Results in Carcinomas of the Breast

Thirty-four patients with carcinoma of the breast were treated; the cases of 29 were evaluable. All 34 patients in this group had had prior treatment of their tumors. Their ages ranged from 33 to 71 years. In 80% of these patients, the primary tumors were resected. In 60%, one to four courses of chemotherapy had been given. Hormone control was attempted in 45%, mainly by oophorectomy or by oral estrogens. The histologic types and responses are listed in table 4. One complete response and two partial responses were observed. None of the responders received any prior hormone therapy and only one patient received thioTEPA as the only prior chemotherapeutic treatment. The only complete response occurred in a 79-year-old white man. His primary tumor had been resected by simple mastectomy 3½ years before receiving treatment with carbestrol. One month later bilateral orchiectomy was carried out for control of pulmonary metastases. One year later he was treated with thioTEPA for reactivation of pulmonary metastases. Three and one-half years after the simple mastectomy his pulmonary metastases became resistant to thioTEPA and he was treated with carbestrol. He received carbestrol for only 3 weeks when it was discontinued because of severe nausea and lymphangitis in his left arm. Five weeks after the beginning of treatment his pulmonary lesions disappeared. He had a complete response for 1 week and an excellent partial response for 5 months while receiving diethylstilbestrol intermittently. He died 8 months later with extensive pulmonary metastases.

TABLE 4.—Responses of patients with carcinoma of the breast to carbestrol

Disease	Evaluable	Not evaluable	Total	Response* CR	PR	NC	P
Adenocarcinoma	24	4	28	0	1	16	7
Infiltrating duct cell carcinoma	3	1	4	0	0	3	0
Paget's disease	1	0	1	0	1	0	0
Male adenocarcinoma	1	0	1	1	0	0	0
Totals	29	5	34	1	2	19	7

*CR = complete remission; PR = partial remission; NC = no change; P = progression.

TABLE 5.—Responses of patients with various solid tumors to carbestrol

Disease	Evaluable	Not evaluable	Total	Response*			
				CR	PR	NC	P
Respiratory system							
Squamous cell carcinoma, lung	1	1	2	0	0	1	0
Undifferentiated cell carcinoma, lung	1	0	1	0	0	1	0
Mesothelioma, pleura	2	0	2	0	0	2	0
Totals	4	1	5	0	0	4	0
Gastrointestinal tract							
Adenocarcinoma, pancreas	1	0	1	0	0	1	0
Adenocarcinoma, colon	1	1	2	0	0	1	0
Carcinoid	1	0	1	0	1	0	0
Totals	3	1	4	0	1	2	0
Skin							
Squamous cell carcinoma	1	1	2	0	0	1	0
Melanoma	1	0	1	0	1	0	0
Totals	2	1	3	0	1	1	0
Others							
Adenocarcinoma, prostate	1	1	2	0	1	0	0
Lymphoma	1	0	1	0	0	1	0
Adenocarcinoma, salivary gland	1	0	1	0	0	1	0
Squamous cell carcinoma, cervix	1	1	2	0	1	0	0
Totals	4	2	6	0	2	2	0

*CR = complete remission; PR = partial remission; NC = no change; P = progression.

Results in Other Solid Tumors

Eighteen patients with various solid tumors were treated; the cases of 13 were evaluable. Only one patient did not receive any prior cytotoxic therapy. The ages of the 18 patients ranged from 43 to 79 years. The histologic types and responses are listed in table 5. Partial responses were observed in four patients who had melanoma, carcinoid tumor, squamous cell carcinoma of the cervix, and adenocarcinoma of the prostate gland. The patient with the prostatic cancer did not receive any prior hormonal therapy.

DISCUSSION

Metastatic sarcomas are known to be resistant to chemotherapy; however, tumor regressions have been seen with a variety of standard cytotoxic agents during the last few years.

Vincristine has caused partial or complete responses in three of eight patients with reticulum cell sarcomas (1). The same tumor type was found to be responsive to adriamycin in four of five patients (2). The combination of actinomycin D and vincristine produced six responders in 12 patients with various sarcomas, with a 5.3-month median duration of response (3). The pediatric investigators of the Southwest Cancer Chemotherapy Study Group have studied the antitumor activity of a number of drugs in the treatment of children with metastatic osteogenic and Ewing's sarcomas. Of 22 patients with Ewing's sarcomas, five partial regressions were noted in 13 patients treated with cyclophosphamide and four partial regressions were noted in nine patients treated with daunomycin (4). Similar results are confirmed elsewhere (5). Encouraging results were reported in the treatment of metastatic osteogenic sarcomas with adriamycin, which produced three responses in nine patients treated (2).

It is difficult to compare these figures to the results in the present phase II study of carbestrol because of the wide variety of sarcoma types in the present study. There were four responses noted in 38 sarcoma patients (10% response rate) in the present study. Only one of these four responders had had prior chemotherapy.

The many different agents and combinations being investigated for the control of advanced mammary carcinoma during the last 2 decades show considerable variation in results. Remission rates range from 14% to 75% (6). The following figures are taken from studies where a 50% decrease in measurable lesions was the basic criterion for a partial response. In the Memorial Hospital for Cancer and Allied Diseases, New York, New York, 337 women with primary, advanced, or metastatic cancer were treated systemically during a 20-year period with various chemotherapeutic agents (6). Fifteen percent of these patients experienced objective remissions for 2 months and 10% for 6 months. Methotrexate produced the highest number of objective responses, with chlorambucil, thioTEPA, vinblastine, 5-fluorouracil, cyclophosphamide, and nitrogen mustard producing fewer objective responses in that order. Response rates for hormone treatment in the same study were 22% with estrogen, 26% with androgens, and 25% with corticosteroids. Similar response rates are reported elsewhere (10–13). The overall response rate in the present phase II study of carbestrol was 10% in breast cancer. None of the responders received prior hormone therapy.

On the basis of our experience we must conclude that carbestrol is of little value in the treatment of patients with sarcomas who have had extensive prior cytotoxic treatment and in patients with mammary cancers who have had prior estrogen treatment. In this study, the results in the remaining patients with solid tumors are based on too small a series for statistical confidence. Further studies with investigational agents should take into consideration that the cases of patients with extensive prior chemotherapy might not be evaluable for drug effectiveness. Since the responses in mammary cancer occurred only in patients who had not received any prior estrogen treatment, a randomized study using carbestrol in patients who had received an estrogen compound without additional cytotoxic effects seems to be warranted.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127

Carbestrol: NSC-19962; CAS reg. No. 1755-52-8; 3-cyclohexene-1-carboxylic acid, 3-ethyl-4-(*p*-methoxyphenyl)-2-methyl-

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Daunomycin: NSC-82151; CAS reg. No. 23541-50-6

Diethylstilbestrol: NSC-3070; 4,4′-stilbenediol, *a*,*a*′-diethyl-

Estradiol: NSC-9895; CAS reg. No. 50-28-2

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Nitrogen mustard: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride

Porfiromycin: NSC-56410; CAS reg. No. 801-52-5; carbamic acid, ester with 6-amino-1,1*a*,2,8,8*a*,8*b*-hexahydro-8-(hydromethyl)-8-amethoxy-1,5-dimethyl-azirino[2′,3′:3,4]pyrrolo[1,2-*a*]indole-4,7-dione

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-; triethylenethiophosphoramide

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; leurocristine, sulfate (1:1)

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Phase II Study of 5-Azacytidine (NSC-102816) in the Treatment of Advanced Gastrointestinal Cancer [1,2,3]

Charles G. Moertel, Allan J. Schutt, R. J. Reitemeier, and R. G. Hahn [4]

SUMMARY

5-Azacytidine was administered to 29 patients with advanced gastrointestinal carcinoma; the total dose level ranged between 500 and 750 mg/m² given in 5- or 10-day courses. Although mild to moderate degrees of leukopenia and mild thrombocytopenia were observed, the dose-limiting toxic reaction was severe nausea and vomiting, sometimes associated with diarrhea. Only one transient and partial objective response was observed.

[Cancer Chemother Rep 56:649–652, 1972]

5-Azacytidine was synthesized by Pískala and Šorm in 1964 (1) as an analog of cytidine. It is incorporated into both RNA (2) and DNA (3) and may exert antineoplastic activity by inhibiting DNA, RNA, and protein synthesis. Its inhibitory action is cell-cycle specific, with cells in the S phase showing the greatest sensitivity (4). 5-Azacytidine has shown significant antitumor effect against L1210 mouse leukemia, with little schedule dependency except that single-dose therapy was inferior to continuous or intermittent administration over a 5–9-day period.[5]

In a phase I study by Weiss and associates (5), the most troublesome human toxic effect of 5-azacytidine was nausea and vomiting, which was related to the amount of drug given in each injection. They found that a total dose exceeding 16 mg/kg (approximately 675 mg/m²) would produce a rather marked hematologic depression. Of special pertinence to this paper is that objective remissions were reported in two of six patients with colon cancer.

The present study was undertaken as a phase II investigation of 5-azacytidine in the treatment of patients with advanced gastrointestinal cancer, particularly cancer of the large bowel.

MATERIALS AND METHODS

We selected 29 patients for study, all of whom had histologic confirmation of unresectable adenocarcinoma of the gastrointestinal tract. All were ambulatory outpatients maintaining a reasonable state of nutrition, and all had measurable areas of known malignant disease to serve as an objective indicator of response.

There were 12 men and 17 women. Their mean and median age was 55 years with a range of 31–72 years. Of the 29 patients, 27 had primary cancers in the large bowel, one had a primary pancreatic cancer, and one had a primary gastric cancer.

Fourteen patients had had no previous treatment of their advanced cancer, 15 had had previous chemotherapy (primarily with 5-fluorouracil [table 1]), and five had had previous radiation therapy. No patients, however, had had either radiation therapy or chemotherapy

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[1] Received Apr 10, 1972; revised Aug 2, 1972.

[2] Supported by contract NIH 70-2066 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health.

[3] 5-Azacytidine was supplied by the Cancer Therapy Evaluation Branch, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Division of Gastroenterology and Internal Medicine (Drs. Moertel, Reitemeier, and Schutt) and Division of Clinical Oncology and Internal Medicine (Dr. Hahn), Mayo Clinic and Mayo Foundation, Rochester, Minn.

[5] Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816), Oct 1970. Prepared by the NCI, Bethesda, Md.

TABLE 1.—Previous treatment experienced by patients in the 5-azacytidine study

Prior treatment	No. of patients
None	14
Chemotherapy	15
5-Fluorouracil	15
CCNU	6
NSC-82196	5
Cyclophosphamide	3
Bleomycin	2
Camptothecin	2
BCNU	1
Radiation therapy	5

within 1 month of entry into the study; all had normal leukocyte and platelet counts.

5-Azacytidine was reconstituted in sterile water and administered by rapid intravenous (iv) push immediately thereafter. We employed three dose schedules: the first, a daily injection for 5 days; the second, a daily injection for 10 days; and the third, injections twice daily for 5 days. The total dose for a course, regardless of the schedule, ranged between 500 and 750 mg/m². Twenty-two patients were given total doses of 750 mg/m² in their first courses; the total doses of three of the remaining seven patients were raised to this level in their second courses.

Patients were routinely given 10 mg of prochlorperazine (Compazine) hypodermically, immediately prior to each injection of 5-azacytidine. Additional prochlorperazine was given throughout the day as needed, either hypodermically or by rectal suppository.

All patients had full hematologic, hepatic, and renal function studies before treatment and on the day following each course of 5-azacytidine. Leukocyte and platelet counts were obtained twice weekly thereafter. All cases were re-evaluated at 5 weeks, and those patients who were physically able were treated again. If a patient had not shown progression at 10 weeks, treatment was continued at 5-week intervals until progression was evident.

RESULTS

Toxic Reactions

Table 2 shows the toxic reactions experienced by our 29 patients to their first course of therapy. Nausea and vomiting were ubiquitous and very troublesome problems. Patients began vomiting between ½ and 3 hours after receiving an injection of 5-azacytidine and continued for periods ranging from 3 to 10 hours. Phenothiazine antiemetics, even when given in very large doses, were not noticeably effective in alleviating these symptoms. Frequently, a flurry of loose stools would accompany the retching. These symptoms occurred only on the days the drug was administered. Most patients lost weight due to these symptoms. The weight loss ranged between 2 and 13 pounds over the 5–10-day course. Several patients required parenteral hydration. One patient refused to continue therapy after experiencing violent retching and diarrhea with the first dose. This patient is not included in any of the other data presented. To put this problem into perspective, we found 5-azacytidine to be a far more potent emetic agent than either NSC-45388 or NSC-82196.

TABLE 2.—Toxic reactions of patients to first course of 5-azacytidine therapy

Toxic reactions	No. of patients
Nonhematologic (29 patients)	
Nausea	28
Vomiting	27
Diarrhea	11
Dermatitis	1
Hematologic (28 patients)	
Leukocyte count $< 4000 > 2000$ cells	15
< 2000 cells	3
Thrombocyte count	
$< 150{,}000 > 100{,}000$	8
$< 100{,}000$ cells	0

We did not observe any stomatitis. One patient had transient and mild maculopapular dermatitis following each of two courses. There were no other late gastrointestinal or mucocutaneous reactions. Of our patients, 68% showed some evidence of hematologic toxicity. This was usually mild and transient, and there was no instance of life-threatening toxicity. The mean nadir of leukopenia was 25.5 days with a range of 18–30 days. The mean nadir of thrombocytopenia was 18 days with a range of 13–21 days. Cumulative hematologic toxicity with repeated courses was not detected.

There were only two possible instances of hepatotoxicity. One patient had a transient rise in his alkaline phosphatase level and the other patient had a transient, mild rise in his serum glutamic oxaloacetic transaminase level (SGOT). It seemed likely, however, that these effects could have been due to the large doses of phenothiazines we had used in an attempt to alleviate the vomiting.

Changing from one schedule of administration to another was done primarily to aid the patients in tolerating the vomiting problem. Table 3 shows the severity of vomiting the patients experienced with the first course given in the three schedules we tried. The single injection for 5 days was clinically unacceptable. Twelve of 12 patients had severe and disabling vomiting each day of treatment. The 10-day schedule was a little better, but for most patients it just meant they were in trouble for 10 days instead of 5 and they lost a little more weight. The least troublesome schedule was twice daily for 5 days. Although it is doubtful that with a larger number of patients treated with this schedule the vomiting would be any less severe than with the 10-day schedule, at least it is over in half the time.

The degree and pattern of hematologic toxicity seemed comparable between the 5-day single-dose and twice daily methods. Among ten patients initially treated with a total dose of 750 mg/m² over 10 days, only five of them had leukopenia, compared to nine of 11 when this same total dose was given in 5 days. This finding of increased reactions with a shorter total period of administration is comparable to that recorded by Weiss and associates (6). As might be expected, the mean nadir of leukopenia occurred 2½ days later with the 10-day course than with the 5-day course.

TABLE 3.—Severity of vomiting and schedule of administration for 5-azacytidine therapy

	Administration schedule		
Grade of vomiting*	Once daily × 5 days	Once daily × 10 days	Twice daily × 5 days
4	12	5	1
3		3	3
2		2	4
1			2
0		1	1

*0 = No vomiting; 4 = severe and disabling vomiting each treatment day.

RESULTS

The therapeutic results of 28 patients treated with at least one full course of 5-azacytidine are documented in table 4. Our criterion for objective response was a 50% decrease in the product of the longest perpendicular diameters of the most clearly measurable area of known malignant disease in the absence of an increase in any other lesions or the appearance of new lesions. We observed only one partial response which lasted for only 5 weeks. No objective responses were observed at 10 weeks. This therapeutic activity is therefore significantly inferior to that observed with 5-fluorouracil, which we and most investigators have noted produces objective responses lasting for 2 months or longer at about a 20% response rate. If we were to stretch our criteria to include the more equivocal 25% decrease in tumor size, there would be two additional responses at 5 weeks and one of these was maintained for 10 weeks. If we look at other parameters, such as performance status and body weight, the results are equally dismal.

TABLE 4.—Therapeutic results in 28 patients treated with 5-azacytidine

	Patient status at—					
	5 weeks			10 weeks		
Parameter of response	Improved	Stable	Worse	Improved	Stable	Worse
			No. of patients			
Objective response*	1	8	19	—	3	25
Weight change†	1	8	15			
Performance status‡	1	11	16			

*Improved = > 50% decrease in tumor size (*see* text for discussion); stable = no therapeutic response; worse = disease progression.

†Improved = gain > 2.5% of pretreatment weight; stable = ± 2.5% of pretreatment weight; worse = loss > 2.5% pretreatment weight. Weight at 5 weeks could not be determined in four patients.

‡Improved = 20% or greater (Karnofsky scale [9]); stable = 20% (Karnofsky scale); worse = 20% or greater (Karnofsky scale).

DISCUSSION

Although this trial was not controlled by other modes of therapy for patients with gastrointestinal cancer, it is our belief that the severity of nausea and vomiting induced by 5-azacytidine seriously compromises any hope of clinical usefulness. Since we were unable to demonstrate any meaningful clinical activity in 27 adequately treated patients with cancer of the large bowel, and since preclinical studies do not indicate the likelihood that pursuit of alternative dose schedules would be fruitful, it seems doubtful that further investigation of 5-azacytidine for this neoplasm is justified.

LIST OF COMPOUNDS

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Camptothecin: NSC-100880; CAS reg. No. 25387-67-1

CCNU: NSC-79037; CAS reg. No. 13010-47-4; 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-; imidazole carboxamide; DTIC

NSC-82196: CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-; TIC mustard

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Combination Therapy With 5-Fluorouracil (5-FU; NSC-19893) and 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962) for Disseminated Gastrointestinal Carcinoma [1,2,3]

Jacob J. Lokich and Arthur T. Skarin [4]

SUMMARY

A total of 20 patients with widely disseminated gastrointestinal cancer (carcinoma of the pancreas in five patients, carcinoma of the stomach in four, and carcinoma of the colon in 11) were treated with a combination of 5-fluorouracil (5-FU; 10 mg/kg/day) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; 40 mg/m^2/day). The drugs were given concurrently by the intravenous route for 5 consecutive days every 6–8 weeks. Objective responses were observed in three of four patients with evaluable cases of carcinoma of the pancreas, in one of eight patients with evaluable cases of carcinoma of the colon, and in none of the three patients with evaluable cases of carcinoma of the stomach. The median survival time of the responding patients was 52 weeks compared with 12 weeks for nonresponding patients. Significant toxicity was encountered in three patients: one had severe thrombocytopenia, one had pancytopenia, and one had irritation of the gastrointestinal tract. The combination of 5-FU and BCNU may be particularly promising in the therapy for carcinoma of the pancreas, but it appears to have limited usefulness in the treatment of carcinoma of the colon.

[Cancer Chemother Rep 56:653–657, 1972]

Improvement in the effectiveness of the chemotherapy for disseminated carcinoma with the use of combinations of drugs is determined by the efficacy of each agent used singly and by the inherent sensitivity of the tumor. The response rate for patients with disseminated gastrointestinal cancer treated with 5-fluorouracil (5-FU) alone is approximately 18% (1); the response rate for patients treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) alone is 13% (2). Reitemeier et al treated patients with a combination of 5-FU and BCNU and obtained objective responses in 22% of their patients. These investigators indicated that carcinoma of the pancreas and carcinoma of the stomach were especially responsive to treatment with this combination (3). Our report confirms both the possible sensitivity of carcinoma of the pancreas and the resistance of carcinoma of the colon to this combination of drugs.

MATERIALS AND METHODS

Twenty patients with gastrointestinal carcinoma (carcinoma of the pancreas in five patients, carcinoma of the stomach in four, and carcinoma of the colon in 11) requiring treatment for disseminated disease between September 1970 and December 1971 were selected for

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[1] Received Apr 12, 1972; revised June 1, 1972.

[2] Supported in part by research grant CA-6516 from the National Cancer Institute (NCI), National Institutes of Health, Public Health Service.

[3] 5-FU: CAS reg. No. 51-21-8.
BCNU: CAS reg. No. 154-93-8.
Supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment (formerly Chemotherapy), NCI.

[4] Children's Cancer Research Foundation, Inc., Peter Bent Brigham Hospital, and Harvard Medical School, Boston, Mass.

therapy with the combination of 5-FU and BCNU. For each patient, metastasis was documented on the basis of abnormal results of either liver scan, bone series or chest roentgenograms, or laparotomy with gross observation of the tumor and biopsy. Only patients who were expected to live at least 1 month were accepted for treatment. Seventeen of the 20 patients had measurable lesions. Two patients had received local radiation therapy previously for metastasis to surgical incisions but no patient had had extensive radiotherapy or prior chemotherapy. Chemotherapy was started within 1 week of laparotomy and diagnosis unless a major anastomosis was performed. If a resection was necessary, a minimum of 4 weeks was allowed to elapse before chemotherapy was started since both 5-FU and BCNU have a toxic effect on the gastrointestinal tract.

Patients were treated on an outpatient basis. Both drugs were administered for 5 consecutive days at 6–8-week intervals. 5-FU (10 mg/kg/day) was given intravenously (iv) in bolus form on Days 1–5. BCNU (40 mg/m^2/day) was given iv concurrently as a 30-minute infusion to minimize the effect of the diluent alcohol and to obviate gastrointestinal toxic effects. Antiemetics were administered daily before the infusion. The dosage was modified as follows: if the white blood cell count (wbc) was 3000–4000 cells/mm^3, the platelet count 75,000–100,000 platelets/mm^3, or the hematocrit value less than 30%, the dose was reduced by one half. Treatment with the drugs was withheld if the wbc was less than 3000 cells/mm^3, the platelet count less than 75,000 platelets/mm^3, or the hematocrit value less than 25%.

Complete blood cell counts were obtained twice weekly for 2 weeks and thereafter at 2-week intervals prior to the start of a second course of treatment with the combination of 5-FU and BCNU. A complete evaluation including barium contrast studies, bone series, chest roentgenograms, liver scan, and complete physical examination was performed after a patient had received two complete courses of therapy.

Objective responses were defined as complete if all measurable lesions disappeared for at least 1 month and partial if there was a reduction in lesion size of 50% or more lasting for not less than 1 month. Chemotherapy was discontinued after two courses if no effect was observed or sooner if tumor progression occurred. If an objective response was achieved, the two agents were administered continuously every 6–8 weeks.

RESULTS

The patterns of metastatic disease and the response and survival time of the patients in each tumor group are shown in table 1. Four of the 15 patients in whom response could be evaluated responded to treatment: three of these patients had carcinoma of the pancreas and one had carcinoma of the colon. The latter patient had a partial response after one course of treatment but was lost to followup study. The cases of the three patients with carcinoma of the pancreas are noteworthy and will be summarized in detail (*see* Case Reports).

Eleven patients who did not respond to treatment died with disseminated tumor within 6 months of therapy. None of these patients had received more than two courses of combination chemotherapy. The median survival time after the start of therapy was 12 weeks (range, 3–28 weeks). Two additional patients are not considered evaluable because of the development of toxicity which either precluded further chemotherapy or was associated with progressive disease. For three other patients (one with carcinoma of the pancreas and two with carcinoma of the colon), there were no objective parameters for measurement of response, but these patients are alive without symptoms 19+, 20+, and 24+ weeks after the first course of chemotherapy.

Significant toxicity was observed in three patients. Severe thrombocytopenia and gastrointestinal bleeding developed in one patient 1 week after the patient had completed the first course of combination therapy; the patient died of progressive disease before a second course of treatment was started. A second patient was uniquely sensitive to the drugs; this sensitivity was demonstrated by the development of pancytopenia during the initial course of therapy. The occurrence of severe gastrointestinal toxic effects made it necessary to discontinue therapy for a third patient. These gastrointestinal toxic effects were observed in milder form in about half of the patients studied.

Case Reports

Patient No. 1 (*see* table 1).—A 40-year-old man was admitted to the hospital in January

TABLE 1.—Response to treatment with the combination of 5-FU and BCNU in patients with gastrointestinal cancer

Patient No.	Age, sex	Tumor		Total No. of courses	Response*	Survival time (wks)
		Primary site	Metastasis to—			
1	40, M	Pancreas	Intestine and peritoneum	8†	100%	56+‡
2	75, M	Pancreas	Liver	3	75%	52
3	38, M	Pancreas	Liver	2	O	12
4	42, M	Pancreas	Incision	3†	NMD	19+‡
5	54, F	Pancreas	Liver and peritoneum	2†	100%	8+‡
6	64, F	Stomach	Peritoneum	1	O	28
7	70, F	Stomach	Peritoneum	2	O	12
8	62, M	Stomach	Liver and peritoneum	1	O	8
9	64, M	Stomach	Liver	1	NE, T	20
10	68, F	Colon	Intestine	1	50%	Lost to followup study
11	67, F	Colon	Intestine	1	O	6
12	67, M	Colon	Intestine and peritoneum	1	O	8
13	60, M	Colon	Peritoneum	2	O	14
14	76, M	Colon	Liver	2	O	12
15	54, M	Colon	Liver	1	O, T	3
16	46, M	Colon	Liver	2	O	20
17	56, M	Colon	Liver and peritoneum	1	NE, T	8
18	64, M	Colon	Cervical lymph nodes	1	O	12
19	72, M	Colon	Incision, intestine and liver	3†	NMD	24+‡
20	38, M	Colon	Retroperitoneal lymph nodes	4†	NMD	20+‡

*O = no response; NMD = no measurable disease; NE = not evaluable; T = toxicity.
†Therapy continuing.
‡Patient still living.

1969 for treatment of abdominal pain and jaundice. Laparotomy revealed adenocarcinoma of the head of the pancreas and a total pancreatectomy with duodenectomy was performed. Nine months later pain recurred but roentgenographic studies of the upper gastrointestinal tract revealed no abnormalities and no further therapy was undertaken for an additional 12 months. An exploratory laparotomy was performed because the patient had increasing pain. Disseminated tumor was found throughout the abdomen; both the retroperitoneum and the peritoneal surfaces, including the serosa of the small intestine, were involved. Treatment with the combination of 5-FU and BCNU was begun according to the regimen outlined previously and the patient's pain gradually decreased after two courses of therapy. In March 1971, the sudden onset of acute abdominal pain prompted a third laparotomy at which time torsion and volvulus with infarction of the small intestine was found. A primary resection of all but 3 feet of the jejunum and 2 feet of the ileum was performed. Exploration of the rest of the abdomen failed to reveal the presence of tumor. Postoperatively, the patient recovered without incident. Therapy with 5-FU and BCNU has been continued at 8-week intervals without difficulty. It has been 3 years since the tumor was observed initially and 14 months since combination chemotherapy was started; there has been no evidence of recurrence of tumor.

Patient No. 2 (*see* table 1).—Mild diabetes mellitus developed in a 75-year-old man in September 1970. Three months later, he was admitted to the hospital with progressive hepatomegaly and diarrhea. Biopsy of the liver revealed anaplastic carcinoma, and a series of roentgenograms of the upper gastrointestinal tract revealed distortion of the middle portion of the duodenal sweep. Treatment with 5-FU and BCNU was started in March 1971; after one course of treatment, the liver was almost normal in size and the liver scan revealed noticeable improvement. In addition, the symptoms of diarrhea and weight loss disappeared. Treatment was continued at 6–8-week intervals until November 1971, when progressive hepatomegaly recurred. The patient died 1 month later. No postmortem examination was performed.

Patient No. 5 (*see* table 1).—A 54-year-old woman had anorexia and weight loss in December 1971. A mass in the upper left quadrant was thought to be an enlarged spleen but at laparotomy it was evident that tumor had replaced the entire left lobe of the liver. The primary tumor was arising from the pancreas; biopsy of this tissue revealed undifferentiated carcinoma. Postoperatively, two nontender nodules were noted over the patient's scalp. Treatment with 5-FU and BCNU was started in January 1972 and within 4 weeks the palpable intra-abdominal masses as well as the nodules of the scalp disappeared and the patient gained 6 kg in body weight. She is alive 2 months after therapy with no evidence of disease.

DISCUSSION

The use of a combination of chemotherapeutic agents is an attempt to achieve a synergistic antitumor effect with minimal toxicity to the host. The antitumor effect is a result of biochemical blockade of various pathways in the synthesis of essential polymers. Theoretical considerations suggest the need for the use of cell cycle-specific agents (eg, 5-FU), which act primarily during the S phase, with cell cycle-nonspecific agents (eg, BCNU), which act throughout the cellular growth cycle (4).

The efficacy of single drugs (particularly 5-FU) in the treatment of gastrointestinal cancer has been emphasized in many studies (5). More recently, Reitemeier et al used 5-FU, mitomycin C,[5] and BCNU in a controlled prospective study to determine the effectiveness of these drugs when they are used alone and in various combinations. The rationale for the choice of drugs was based partially on the differential nadirs of marrow suppression by the two drugs, since the 5-FU depression occurs within 14 days of treatment and the maximum BCNU effect is delayed to 6 weeks. The results indicated that treatment with the combination of 5-FU and BCNU produced the best objective responses and that the combination was particularly effective against carcinoma of the pancreas (with two of three patients responding) and carcinoma of the stomach (with four of six patients responding).

The overall objective response rate of 22% obtained in the Mayo Clinic study (3) was similar to that achieved in our study. Our results have confirmed the fact that carcinoma of the pancreas may be uniquely sensitive to this combination of drugs. Furthermore, the toxicity of this combination of drugs is acceptable and patients may be treated as outpatients. Further investigation of the combination of 5-FU and BCNU in the treatment of pancreatic neoplasms appears to be justified on the basis of this study.

The lack of response in patients with other forms of gastrointestinal neoplasm, particularly carcinoma of the colon, is reflected in the poor median survival time (3 months) of the nonresponding patients and does not appear to be related to the amount of residual metastatic tumor. The combination of 5-FU and BCNU does not appear to be of value in the treatment of this type of gastrointestinal cancer.

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Regression of Intradermal Malignant Melanoma After Intralesional Injection of *Mycobacterium bovis* strain BCG [1,2]

Larry Nathanson [3,4,5]

SUMMARY

Nine patients with intradermal metastases of malignant melanoma were treated with intralesional injections of *Mycobacterium bovis* strain BCG (bacillus Calmette Guérin). Significant toxic effects included fever, nausea, rash, local skin necrosis, and lymphopenia, leukopenia, or both. Clinically significant systemic BCG infection was not identified. Seven of the nine patients experienced local regression of BCG-treated lesions and two of these seven experienced complete regression of lesions which had not been treated. Four of the responding patients and both of the nonresponding patients had had negative purified protein derivative (PPD) skin tests prior to BCG treatment; conversion to positive reaction to PPD after BCG treatment was noted only in the four patients who responded to treatment. This system may prove to be a reproducible model for the study of immunologically mediated regression of malignant melanoma.

[Cancer Chemother Rep 56:659–665, 1972]

Great interest has been focused in recent years on the immunologic features of cancer in man. Work in our own laboratories (1), as well as in those of Hellström (2), Morton (3), Alexander (4), and others, has demonstrated that sensitivity to human tumor antigens, evidenced by both the presence of circulating antibody and cell-mediated sensitivity, is indeed present. Observations of in vitro cytotoxicity in tumors exposed to autologous lymphocytes raise a new question: namely, why does the in vivo host-mediated immune defense fail to protect the patient against his tumor despite the presence of lymphocytes sensitized to new antigenic determinants on tumor cells? This failure has been attributed to various factors such as the presence of blocking antibody (resulting in tumor "enhancement"), neonatal exposure to antigen (resulting in the development of "tolerance"), and resistance of tumor cells to immunodestruction. This report is concerned with an attempt to overcome whatever inhibitory factor may be present with the use of an immunologic adjuvant. *Mycobacterium bovis* strain BCG (bacillus Calmette Guérin), in association with continued exposure of the host to tumor antigens (5).

MATERIALS AND METHODS

Nine patients were selected for treatment on the following basis: none had evidence of advanced metastatic disease and all had intradermal (id) metastases not amenable to surgi-

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[5] Acknowledgement is given to Dr. Robert S. Schwartz for his critical review of the manuscript.

cal treatment. The BCG used was a strain originated at the Pasteur Institute and produced by the Tice Laboratories of the University of Illinois School of Medicine, Institute for Tuberculosis Research. Another strain (originated in Copenhagen, manufactured by Glaxo Laboratories in Great Britain, and marketed by the Lilly Co. in the United States) was not used in the study because it appeared to produce a somewhat less intense local inflammatory response. The material was prepared in a suitable diluent and 0.1 ml of the preparation was injected id into the base of each melanoma lesion to be treated. The BCG preparation contained approximately 3×10^6 viable colony-forming units or approximately 3×10^7 viable bacteria per cubic centimeter of solution. Up to six nodules were treated during a single visit. Some nodules were left completely untreated and other nodules were treated with saline to serve as controls. Biopsy specimens of the tumor nodules were obtained both before and during the process of regression. Commercially prepared impregnated agar plates (Hyland Laboratory) were used for the determination of complement levels. Complement was measured as the third component only (β_1C).

RESULTS

The sequence of events following the id injection of BCG was fairly constant (figs 1–5). Lesions which had been treated with saline injections (controls) never underwent significant changes. Local inflammation appeared within 2–4 hours at the injection site (fig 2) and persisted for 7–14 days. Fever commenced at about the same time in six patients; however, temperatures higher than 101 F (measured orally) were rare. Leukopenia with absolute lymphopenia developed in four patients and lymphopenia without leukopenia developed in three others. The nadir of the white blood cell count (wbc) occurred usually after three to four weekly BCG injections had been given. In 2–3 weeks, local necrosis and scab formation with slough occurred at the BCG injection site (fig 3); this healed subsequently leaving a minor residual scar. On two occasions, the healing of this ulceration took up to 12 weeks. On one occasion, distant untreated lesions developed halos of inflammation but regressed only partially. In two patients, all untreated lesions regressed also, often leaving crescent-shaped areas of residual pigmentation (fig 4). Injections were continued on a weekly basis for up to 8 weeks (maximum) although most patients received treatment for only 4 weeks. There was a regular tendency for both local inflammatory and systemic symptoms to increase in severity with successive injections. In none of our patients was reaction sufficiently severe to justify antituberculous chemotherapy. We were unable to culture or identify organisms in postinjection sites on four occasions 4–8 weeks after the start of treatment (6). One patient had a widespread erythematous maculopapular rash on a treated lower extremity which resolved over 6–8 months; biopsy of this tissue revealed nonspecific granuloma and no organisms.

Histologic examination (fig 6) of the treated areas 3 weeks after BCG treatment revealed mononuclear cell infiltration (fig 7), areas of giant cell formation (fig 8), and residual pigment in melanophages (fig 9), without any visible residual tumor cells. One year after the start of therapy, complete regression of all lesions persisted (fig 5) in one patient.

Our experience with this technique is summarized in table 1. In seven of the nine patients, response occurred at least at the site of BCG injection. Responding patients (ie, those patients in whom treated lesions decreased in size by more than 50% for more than 1 month) and nonresponding patients do not appear to differ significantly in age. Seven of the nine patients were women. Each patient had a primary lesion in either the foot or the leg. All patients had id disease and, in addition, all of the nonresponding patients and almost half of the responding patients had clinical evidence of local or regional lymph node involvement; for most patients, recurrence was noted after regional node dissection. Distant metastases were present in one of the seven patients who responded and in one of the two patients who failed to respond. Two of the responding patients and both of the nonresponding patients had undergone chemotherapy prior to treatment with BCG. The time from diagnosis to the start of therapy was significantly longer in the responding patients than in the nonresponding patients and responding patients survived longer. The two surviving patients had complete responses. All of the patients (with one exception) had local inflammatory reactions at the

TABLE 1.—Data on patients with melanoma treated with BCG

	Patients responding to treatment	Patients not responding to treatment
No. of patients treated	7	2
Men	2	0
Women	5	2
Median age	56	42
Primary site of tumor		
Foot	3	2
Leg	4	0
Extent of metastasis		
Intradermal	7	2
Lymph nodes	3	2
Distant	1	1
Prior treatment		
Radiation therapy	1	2
Chemotherapy	2	2
Interval between diagnosis and start of treatment (mos)	40	24
Interval between start of treatment and death (mos)	14+ (2 patients alive)	4
Toxic effects		
Local inflammation	7	1
Fever	6	1
Leukopenia	4	0
Complement (β_1C; mg/100 ml)		
Before treatment	1.4	1.2
After treatment	0.9	1.0
PPD skin test*		
Before treatment		
Negative	4	2
Positive	3	0
After treatment		
Negative	0	2
Positive	7	0
Response to treatment		
Treated lesions	7	1§
Untreated lesions		
Flare§	1	0
Regression	2†	0
Mean duration of response (mos)‡		
Partial response	6	—
Complete response	19+	—

*PPD = purified protein derivative.

†Melanogens, detected in the urine of one patient prior to BCG treatment, were undetectable after treatment.

‡*See* text for explanation of response.

§Erythematous halo around lesion but no objective decrease in size.

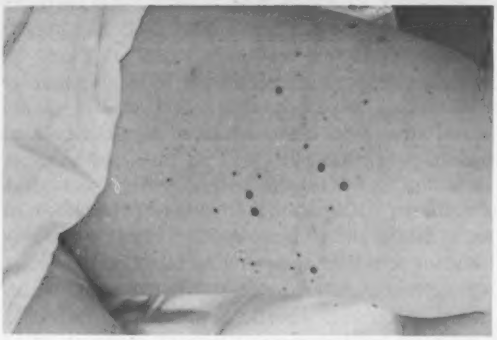

FIGURE 1.—Id metastases in the leg of a 73-year-old woman with melanoma (prior to BCG injection). (This patient is the subject of all subsequent figures.)

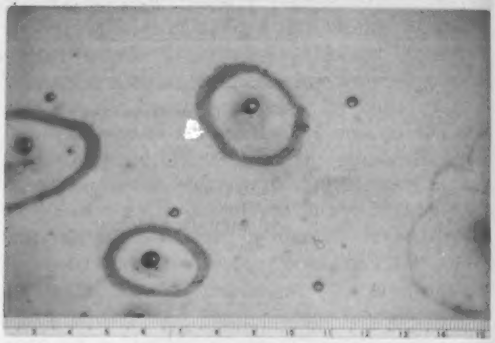

FIGURE 2.—Closeup view of local inflammation 1 week after injection of BCG (0.1 ml) into lesion bases.

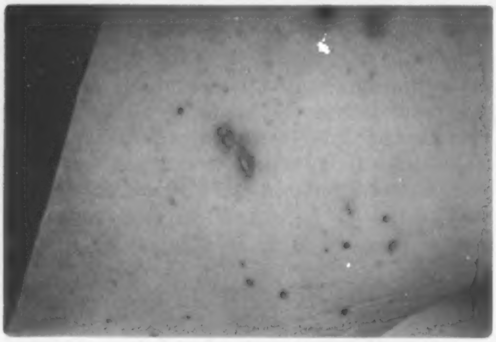

FIGURE 3.—Results of slough of postinflammatory scab with clean healing of tumor-free scar 3 weeks after treatment. Note regression of several untreated lesions.

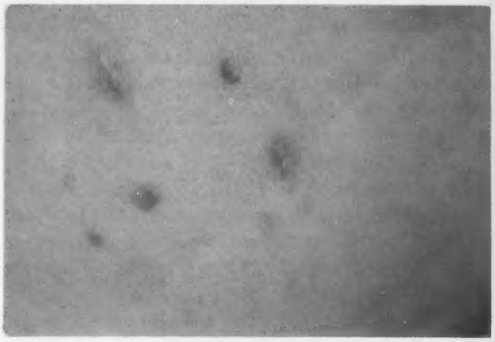

FIGURE 4.—Closeup view (6 weeks after treatment) of two healed BCG-treated lesions and regression of two untreated lesions leaving crescent-shaped areas of residual pigmentation.

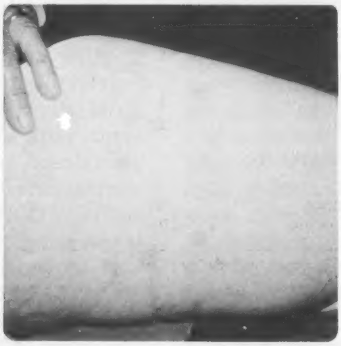

FIGURE 5.—Patient's leg 12 months after start of BCG therapy.

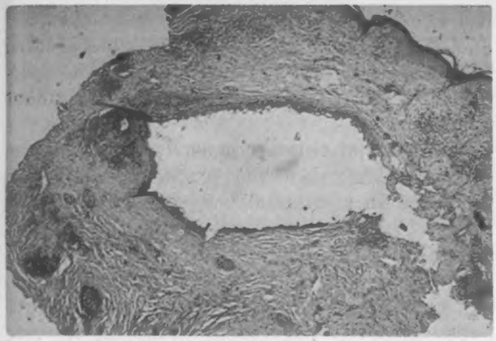

Figure 6.—Biopsy section of regressed id melanoma 3 weeks after BCG injection. Note space presumably occupied by tumor nodule prior to treatment. No viable tumor cells are visible.

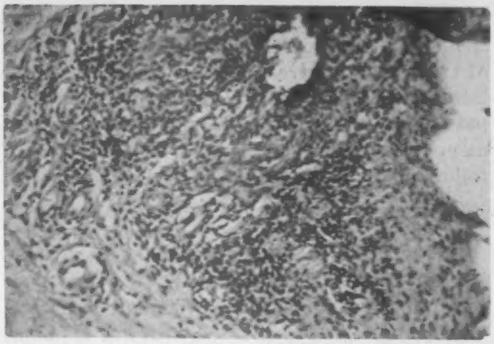

Figure 7.—Higher magnification of biopsy section (fig 6). Note perivascular distribution of mononuclear cell infiltration.

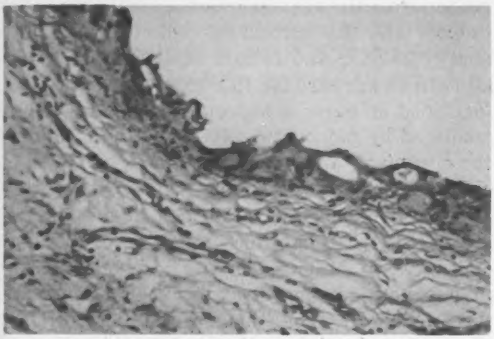

Figure 8.—Higher magnification of edge of space visible in biopsy section (fig 6). Note giant cell formation.

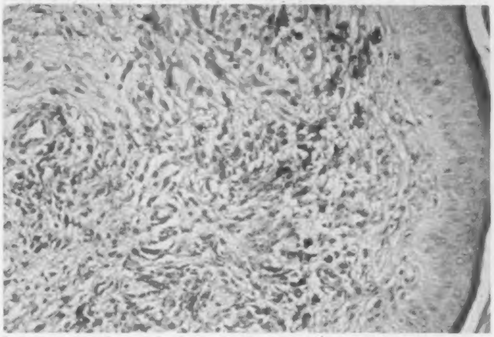

Figure 9.—Higher magnification of epidermal area in biopsy section (fig 6). Note pigment in melanophages.

sites of injection. Most also experienced some degree of fever and two had moderate, but not severe, leukopenia which was always accompanied by lymphopenia (nadir count, 2500 cells/mm^3). Complement levels fell consistently to a lower (but not statistically significant) level in the responding patients after BCG treatment. This degree of variation in β_1C levels was not seen in a comparable group of untreated patients with melanoma. Four of the seven responding patients had had negative purified protein derivative (PPD) skin tests prior to therapy; all had positive PPD tests after therapy. Both of the non-responding patients had had negative PPD tests prior to therapy; however, neither patient had a positive reaction after therapy. This suggested a relative state of anergy in the nonresponding patients which may have been related to their failure to respond. One of the "nonresponding" patients did experience a local flare reaction but significant regression of BCG-treated tumor nodules did not occur. In two responding patients, untreated lesions regressed by more than 50%. One of these patients had had detectable urinary melanogens prior to BCG treatment; the disappearance of these melanogens after treatment suggested regression of systemic disease (7). It should be emphasized that useful clinical palliation of both bleeding and draining local lesions was achieved in several of these patients. The mean duration of response was 6 months in patients who had partial responses (regression of treated lesions only) and more than 19 months in patients who had complete responses (regression of treated and untreated lesions). One of these latter patients is still in remission. The other patient had a second remission with BCG treatment after relapse occurred. The duration of followup study ranged from 8 to 24 months.

DISCUSSION

In 1959, Old and Clarke reported the results of their work with Sarcoma 180 (8). The tumor was implanted in animals who had been previously infected with BCG. Death due to tumor was significantly delayed by this pretreatment. In a similar experiment they demonstrated also a delay in growth and decreased mortality from Carcinoma 755 implanted after BCG injection. In 1961, Weiss published data obtained in studies of T-32 mammary carcinoma showing that the rate of growth of this tumor was decreased by pretreatment with either living BCG organisms or a methanol extract of BCG (9). This extract was more effective than a phenolized BCG preparation. In 1970, Zbar and Rapp showed that BCG can have some antitumor activity when it is injected alone. This action was strikingly enhanced when BCG was mixed with a living line of tumor cells and injected after prior tumor implantation (10). The immunity conferred was specific: immunity to id growth of a challenge inoculum of line 10 tumor cells was not observed in animals immunized with a mixture of BCG and line 1 tumor cells. In 1968, Mathé showed that increases in the survival time of mice bearing L1210 leukemia could be achieved with the use of BCG (11). If an inoculum of more than 10^5 tumor cells was used, no protective effect was afforded by the BCG inoculation. The further demonstration by Simmons that the immunospecific rejection of methylcholanthrene-induced fibrosarcoma with the use of neuraminidase-treated tumor cells can be magnified by the use of BCG is also of great interest (12).

Thus, this series of experiments suggests (*a*) that BCG may be protective against tumors when it is inoculated either before or after tumor transplantation; (*b*) that the benefit afforded by BCG is enhanced by the addition of tumor cells; and (*c*) that the enhanced BCG effect is specific for the type of tumor cell injected and may be limited to a maximum body burden of tumor cells. Encouraged by the clinical experiences of Mathé with leukemia (13) and Morton with melanoma (14), we decided to treat a series of patients with direct injections of BCG into id nodules of malignant melanoma.

We felt that this approach would fulfill the following criteria: (*a*) patients would be treated with a minimal body burden of tumor cells; (*b*) the adjuvant would have the potential of close contact and possible conjugation with tumor antigens; and (*c*) the skin, an organ well known for its ability to demonstrate hypersensitivity phenomena, would be the site of immunologic adjuvant exposure.

Our results do, indeed, suggest that an immunologic process was initiated. The local inflammatory process with polymorphonuclear and then mononuclear cell infiltration, together with the regression of distant untreated lesions, and the correlation between apparent anergy to BCG and failure of antitumor effect, all furnish support for this view. Rosenthal has identified an early nonspecific response to BCG mediated by monocytic cells which phagocytose the organisms and a later, immunologically specific response mediated by lymphocytes (15). Lymphopenia, usually identified about 1 week after BCG treatment, may result from this second phase of host response. The host response in our patients appeared to include many of the features of the Arthus reaction which takes place when local injections of soluble antigen are given to animals who possess circulating precipitating antibodies. The early onset of inflammation, apparent drop in complement levels, tissue necrosis, early presence of polymorphonuclear leukocytes, and, later, perivascular mononuclear infiltration and lymphopenia all suggest this possibility. Immunofluorescent microscopy to prove this mechanism was not performed for this series of patients but it is planned for the immediate future.

The toxicity of the therapy seems to be tolerable since no serious residual toxic effects were observed in these patients. These data are consistent with previous reports that BCG is rarely pathogenic in man (16).

The results obtained with this type of adjuvant immunotherapy for malignant melanoma appear to confirm the work of Morton (14) and to give some promise for future study. Because this study lacks suitable control, adequate studies with other possible immunologic adjuvants, including vaccinia virus, and studies comparing the intralesional technique with the scarification and id method of administration, are now being carried out in man to determine the specificity of the BCG effect. We did not study a significant number of patients with metastatic disease; but the finding by Morton (3) that visceral lesions did not respond to treatment suggests a possible limitation of this technique.

Perhaps more important than the therapeutic results achieved is the usefulness of this technique as a model system for the study of presumably immunologically mediated regression of melanoma (17). Further investigations of the presence of "blocking factor" or "unblocking factor," as reported by the Hellströms (2), are now underway in our laboratory (18, 19). It is our belief that this system will prove to be a reproducible in vivo experimental model of the "spontaneous regression" phenomenon in malignant melanoma which will enable us to study these factors in man to an extent not heretofore possible.

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5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) in the Treatment of Lymphoma[1,2,3]

Emil Frei III,[4] James K. Luce,[5] Robert W. Talley,[6] V. K. Vaitkevicius,[7] and Henry E. Wilson[8,9]

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) was synthesized as an analog of aminoimidazole carboxamide, an intermediate in purine biosynthesis (1). Its mechanism of action has not been definitively elucidated (*see* Discussion). NSC-45388 is active in a number of experimental transplanted tumor systems including lymphoma-leukemia systems and melanoma (2). Clinical studies have shown that in 20%–30% of patients with metastatic melanoma, objective response has been demonstrated and widely confirmed (3,4).[10] A phase II study of this agent was undertaken by the Southwest Cancer Chemotherapy Study Group in patients with advanced refractory lymphoma based on the clinical studies mentioned above, the agent's activity in experimental lymphomas, and preliminary evidence in phase I studies for activity in lymphoma. Unequivocal evidence for activity has been demonstrated for patients with Hodgkin's disease and suggestive evidence has been observed in patients with lymphosarcoma.

PATIENTS

Sixty-six patients with disseminated lymphoma, almost all of whom had received and were considered refractory to known effective treatment, were entered in the study between July 1968 and October 1971. Of these 66 cases, 47 (71%) were valid for analysis (table 1). The major reason for invalidation was early death. This was defined as death due to the disease within 1 month of the initiation of NSC-45388 treatment.

Major prior chemotherapy was defined as treatment with, and demonstrated refractoriness to, at least two of the agents established to be effective for lymphoma. Major prior radiotherapy consisted of a minimum of 3000 rads to the axial skeleton (mediastinal, para-aortic, and/or pelvic nodes). Of the 47 patients, all but three had had major prior treatment and 17 had had major prior treatment in addition to radiotherapy and chemotherapy (table 2).

TABLE 1.—Patient sample for NSC-45388 treatment

Patient category*	No. of patients
Entered on study	66
Ineligible	3
Invalid	16
Early death	11
Lost to followup	2
Protocol violation	3
Valid for analysis	47 (71%)

*For definitions *see* text (Patients).

Patients with white blood cell (wbc) counts less than 3000 cells/mm^3 and platelet counts less than 100,000 cells/mm^3 were excluded from the study. Similarly, patients with azotemia were excluded. Measurable tumor masses, as evaluated by radiography or palpation, were recorded prior to treatment and at 2-week intervals during remission induction. Wbc counts, platelet counts, and hemoglobin levels were obtained at a minimum of weekly intervals during remission induction.

TREATMENT PROCEDURE

NSC-45388 was administered intravenously in 5-day courses at an initial dose of 250 mg/m^2/day. Courses were repeated at 3-week in-

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[3] NSC-45388 (CAS reg. No. 4342-03-4) was supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI.

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[8] The Ohio State University, Columbus, Ohio.

[9] *Reprint requests to:* The Southwest Cancer Chemotherapy Study Group, 6723 Bertner Dr, Houston, Tex 77025.

[10] Johnson R. Clinical Trials Task Force Meeting, July 31, 1969.

TABLE 2.—Prior treatment in patients with lymphoma receiving NSC-45388

	Disease category				
Prior treatment	Hodgkin's disease	Reticulum cell sarcoma	Lymphosarcoma	Other	Total
None	0	0	0	0	0
Minor	0	3	0	0	3
Major prior chemotherapy	7	8	11	1	27
Major prior radiotherapy	0	0	0	0	0
Major prior chemotherapy and radiotherapy	11	12	14	0	17
Total	18	23	25	1	47

tervals. The dose was adjusted in accordance with myelosuppression. In the absence of myelosuppression from a previous course, the dose was increased from 250 to 300 or 350 mg/m²/day. If myelosuppression occurred, the dose remained the same or was reduced depending upon the severity of myelosuppression. In general, patients received a minimum of two courses of treatment. If after two courses the disease was progressive, in terms of increase in tumor size, the patient was considered a failure and the study was terminated. If after three courses of treatment there was no improvement, the study was discontinued and the patient considered a failure. If tumor regression occurred, treatment was continued until relapse.

Complete remission was defined as the complete disappearance of objective and subjective disease manifestations. A partial remission constituted a decrease greater than 50% in the product of the diameters of measurable tumor masses. The duration of remission was measured from the beginning of partial remission to relapse. Relapse was defined as unequivocal growth of persisting tumor or reappearance of tumor.

RESULTS

NSC-45388 produced an objective response in ten of 18 (56%) patients with Hodgkin's disease. Only one of the ten responses was complete. Six of the ten patients are still in remission; thus, the minimum median duration of remission is 4 months. Three of the patients have remained in remission for over 10 months and the patient who entered complete remission remains in complete remission after 30 months of treatment. None of 13 patients with reticulum cell sarcoma responded. Four of 15 patients with lymphosarcoma responded and one patient with mycosis fungoides responded. The duration of the responses in lymphosarcoma and mycosis fungoides was brief (table 3).

TOXICITY

While a number of toxic manifestations have been ascribed to NSC-45388, most of them occur infrequently and rarely limit the dose. The two major dose-limiting forms of toxicity relate to myelosuppression and nausea and vomiting. The nausea and vomiting is most severe with the first dose of a 5-day course of treatment,

TABLE 3.—Response of patients with lymphoma to NSC-45388

		No. of remissions				
Disease	No. of patients	Complete	Partial	Total	Percent	Duration of remission (months)
Hodgkin's disease	18	1	9	10	56	2,2+,2+,3,3,4,4+, 10+,12+,30+
Reticulum cell sarcoma	13	0	0	0	0	—
Lymphosarcoma	15	1	3	4	27	2,2,2+,3
Mycosis fungoides	1	0	1	1		2
Total	47	2	13	15	32	

but tends to diminish and almost disappear by the third or fourth dose. Antiemetics have not been particularly effective in preventing the occurrence of this manifestation.

The myelosuppression of NSC-45388 tends to be more variable than that of other antitumor agents. In the 47 patients in this study, 20 had no myelosuppression and 12 had mild to moderate myelosuppression. Five had severe myelosuppression (wbc depression to less than 500 cells/mm^3 or platelet count depression to less than 10,000 cells/mm^3 or both) and five patients had protracted myelosuppression. This is defined as myelosuppression persisting beyond 60 days.

DISCUSSION

This study emphasizes the difficulty in evaluating a new agent in the treatment of diseases such as the leukemias and lymphomas when a number of agents of established efficacy already exist. All but three of the patients in this study had had major prior treatment. The advanced nature of the sample of patients entered in the study is even more dramatically emphasized by the observation that 11 of the 65 patients died of their disease within a month following the initiation of NSC-45388 treatment (table 1). While it has not been unequivocally established for lymphoma, there is ample evidence, in both experimental and in clinical systems, that the longer the duration of the disease, the greater the amount of prior therapy (particularly myelosuppressive therapy), and the more extensive the disease in terms of mass, the less responsive it is likely to be to a given modality of systemic therapy. To this extent our observed results for NSC-45388 in patients with Hodgkin's disease probably represent minimum figures and it is possible that if NSC-45388 were employed earlier in the disease, a substantially greater response rate would be produced. It is fortunate for patients with disseminated lymphoma, particularly Hodgkin's disease, that combination chemotherapy is highly effective and new agents (such as the nitrosoureas, adriamycin [NSC-123127], and perhaps bleomycin [NSC-125066]) are substantially effective (5–7). On the other hand, these effective treatments make it difficult to test other new agents (such as NSC-45388) in earlier disease.

Attempts have been made to improve the therapeutic effect of NSC-45388 by modifications in dose and schedule and by combination chemotherapy. In experimental systems and in man, it has been found that the therapeutic index is not influenced by dose schedule (4). Since NSC-45388 is non-myelosuppressive in the majority of patients studied, it has been combined with myelosuppressive antitumor agents such as adriamycin. The combination of NSC-45388 and adriamycin is synergistic in experimental systems and does not produce additive toxicity.[11] Similarly, in man it has been demonstrated that NSC-45388 and adriamycin may be used in combination at full doses with little or no additive toxicity (8). There is preliminary evidence that this combination produces a significantly higher response rate than either agent alone in patients with soft tissue sarcomas (9). Since both agents are effective in Hodgkin's disease, this combination regimen involving NSC-45388 might be considered in the treatment of Hodgkin's disease.

The activity of NSC-45388 in patients with Hodgkin's disease is consistent with the activity in transplanted leukemia-lymphoma systems in rodents. NSC-45388 has also been effective in patients with metastatic melanoma and soft tissue sarcoma (3). In melanoma we observed a 36% response rate in women as compared to a 14% response rate in men ($P = 0.05$) (3). Of the 18 patients with Hodgkin's disease in this study, 14 were men, of whom seven responded. There were only four women and three of these responded. Of the three longest responses, two were in women.

It may be concluded that NSC-45388 is not active in patients with reticulum cell sarcoma and is minimally and probably not significantly active in patients with lymphosarcoma.

The activity of NSC-45388 in patients with Hodgkin's disease refractory to known effective agents indicates that its mechanism of action is fundamentally different from these agents. It is metabolized in part by aminoimidazole carboxamide (10). It is also metabolized in part by hepatic microsomal enzymes to the highly unstable monomethyl imidazole carboxamide triazeno with the liberation of a methyl carbonium ion which alkylates the 7 nitrogen moiety of guanine in DNA (11,12). While this sequence of events has been conclusively demonstrated in man, it remains to be determined whether the biologic effect and particularly the antitumor effect is related to these biochemical events. The pharmacologic action of NSC-45388 in animals and in man has been extensively studied (13).

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[11] Golding A, and Schabel FM, Jr. Personal communication.

In summary, NSC-45388 represents a new agent of established efficacy in the treatment of patients with advanced Hodgkin's disease. Consideration should be given to its incorporation into therapeutic regimens developed for the treatment of earlier disseminated disease.

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Clinical Experience With 5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (NSC-82196) in the Treatment of Metastatic Malignant Melanoma[1,2,3]

Geoffrey Falkson, A. M. van der Merwe, and H. C. Falkson[4]

SUMMARY

5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide was given intravenously to 20 patients who had metastatic malignant melanoma. The side effects were fever, flushing, malaise, thrombophlebitis, nausea and vomiting, and hematopoietic depression. Of the 20 patients, one had complete remission, two had partial remission, and one showed improvement. None of the remissions were of long duration.

[Cancer Chemother Rep 56:671–677, 1972]

The synthesis of 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (NSC-82196) was first reported by Shealy and Krauth (1). The triazenoimidazole carboxamides were designed as inhibitors of purine nucleoside synthesis (2). An unstable intermediary in the reaction of 5-aminoimidazole carboxamide to hypoxanthine, namely 5-diazoimidazole carboxamide, had been found to inhibit in vitro tumor systems (3). Subsequently, a variety of triazenoimidazole carboxamides were prepared to find an analog with a favorable therapeutic index. The first of this series was the dimethyl derivative, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388). The 2-chloroethyl derivative, NSC-82196, was shown later to be even more active. NSC-82196 has marked activity against L1210 murine leukemia (1) and was also reported active against Walker 256 carcinosarcoma, Sarcoma 180, and Adenocarcinoma 755. Data obtained by the Drug Evaluation Branch, DR&D, DCT, NCI, have shown this activity to be affected by the route and schedule of drug administration (Carter SK, personal communication).

Disseminated malignant melanoma has proved to be refractory to most conventional forms of antineoplastic chemotherapy, although various antimetabolites and alkylating agents have yielded a 6%–15% objective response rate (4,5) and various reports on NSC-45388 have claimed objective responses in approximately 20% of a number of patients with melanoma (6–9). Clinical experience with NSC-82196 given orally has shown discrepancies in toxicity and therapeutic efficacy due to erratic absorption.[5]

A parenteral form of NSC-82196 is now available. In a phase I study, Luce reported five regressions among 16 patients with malignant melanoma and Bagley reported no responses in 12 patients with melanoma treated at the National Cancer Institute.[6] In these studies, single

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[2] Supported in part by a grant from the National Cancer Association of South Africa.

[3] NSC-82196 was supplied by Drug Research and Development (DR&D), Division of Cancer Treatment (DCT), National Cancer Institute (NCI). Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the list of compounds at the end of the text.

[4] Department of Cancer Chemotherapy, H. F. Verwoerd Hospital, and the University of Pretoria, South Africa.

[5] Clinical brochure: 5-[3,3-bis(2-chloroethyl)-1-triazeno)imidazole-4-carboxamide] (TIC mustard, NSC-82196), Jan 1970, p 23. Prepared by the NCI, Bethesda, Md.

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TABLE 1.—Patients with metastatic

Patient No.	Age, sex	Primary site of disease	Area of skin disease	Metastases at start of NSC-82196 treatment					Patient status*
				Lymph gland	Soft tissue	Lung	Liver	Bone	
1	60, M	Unknown			Right axilla	+	+		2
2	51, M	Left foot		Abdominal gland	Peritoneum	+	+	+	2
3	45, F	Left heel		Left inguinal gland		+			2
4	23, M	Unknown	Multiple subcutaneous nodules	Axillary and supraclavicular glands		+	+	+	1
5	41, M	Left heel	Leg	Thigh gland					2
6	67, M	Left neck	Abdomen	Abdominal and inguinal glands			+	+	3
7	44, M	Left upper arm		Left axilla		+			1
8	42, M	Left knee				+	+		4
9	46, M	Left groin	Right breast area	Right inguinal and axillary nodes					1
10	65, F	Left foot				+		+	1
11	55, F	Right hip		Left supraclavicular lumbar area		+			1
12	63, M	Left chest		Left axilla and chest		+			1
13	59, F	Right buttock	Neck, chin, temporal area, chest, abdomen, back, axillae			+			1
14	51, M	Right thigh		Right iliac and inguinal area					1
15	61, M	Right leg	Right ankle and lower leg	Iliac glands					1
16	43, M	Left foot	Anterior abdomen, left thigh and buttock						1

malignant melanoma treated with NSC-82196

Previous treatment	Total dose of NSC-82196	Response†	Toxic effects‡					
			Nausea	Vomiting	Malaise	Lowest hemoglobin level (g/100 ml)	Lowest wbc (cells/mm³)	Lowest platelet count (cells/mm³)
Surgical treatment; radiotherapy; methotrexate	1500 mg/day × 5 (7500 mg)	PD	+		++			
Surgical treatment	1800 mg/day × 5 (9000 mg)	PD	+++	++	+++			60,000 for 20 days
Surgical treatment	1600 mg/day × 10 (16,000 mg; 2 courses)	PD	+++	++	+			
Surgical treatment; vinblastine; chlorambucil	1600 mg/day × 5 (8000 mg)	PD	+++	++	+		1500 for 48 days from Day 16	60,000 for 21 days from Day 29
Surgical treatment; melphalan (iv)	2000 mg/day × 5 (10,000 mg)	PD	+					
Surgical treatment; radiotherapy	1800 mg/day × 5 and 1500 mg/day × 5 (15,000 mg; 2 courses)	Imp (3 mos)	+++	++	+	9.8 on Day 49	1900 on Day 18 for 31 days	77,000 on Day 35 for 13 days
Surgical treatment	1800 mg/day × 10 (18,000 mg; 2 courses)	PD	+++	++	+++	8.4 on Day 50		
Surgical treatment	1800 mg/day × 5 (9000 mg)	PD	++	++	++	8.8 on Day 11		
Surgical treatment	1600 mg/day × 10 (16,000 mg; 2 courses)	CR (2 mos)	+++	++				
Surgical treatment	1600 mg/day × 5 (8000 mg)	PD	+	+		7.8 on Day 28	2300 on Day 18	
Surgical treatment; radiotherapy	1500 mg/day × 5 (7500 mg)	PD	+++	+++			3400 on Day 68	
Surgical treatment	1800 mg/day × 9 (16,200 mg; 2 courses)	PD	++	+	+			
Surgical treatment	1700 mg/day × 5 (8500 mg)	PD	+	+				
Surgical treatment	2000 mg/day × 14 (28,000 mg; 3 courses)	PR (lesions regressed more than 50%; 2 mos)					3500 on Day 110 for 42 days after 2nd course	75,000 on Day 21 for 33 days; 41,000 on Day 31 after 2nd course for 11 days
Methotrexate; vinblastine; chlorambucil; NSC-45388	1800 mg/day × 15 (27,000 mg; 3 courses)	PD	++	++			2900 on Day 26; 1800 on 8th day after 2nd course for 10 days; 3000 on 31st day after 3rd course	82,000 on Day 120
Surgical treatment; 5-fluorouracil (intra-arterial); methotrexate;	1500 mg/day × 10 (15,000 mg; 2 courses)	PD	+	+			3900	99,000

TABLE 1.—Patients with metastatic malignant

Patient No.	Age, sex	Primary site of disease	Area of skin disease	Metastases at start of NSC-82196 treatment					Patient status*
				Lymph gland	Soft tissue	Lung	Liver	Bone	
17	41, F	Back	Chest wall	Left axilla					0
18	52, M	Left arm		Axillary and mediastinal glands			+		1
19	44, M	Left lower leg		Inguinal and abdominal glands			+		2
20	40, F	Back				+	+		2

*0 = Normal; 1 = ambulatory but unable to work; 2 = less than 50% of time in bed; 3 = more than 50% of time in bed; 4 = bedridden.

melanoma treated with NSC-82196—Continued

Previous treatment	Total dose of NSC-82196	Response†	Toxic effects‡					
			Nausea	Vomiting	Malaise	Lowest hemoglobin level (g/100 ml)	Lowest wbc (cells/mm^3)	Lowest platelet count (cells/mm^3)
melphalan; procarbazine; BCNU + vincristine; NSC-45388; bleomycin; hydroxyurea + radiotherapy								
Surgical treatment; procarbazine	1300 mg/day × 15 (19,500 mg: 3 courses)	PR (1 mo)	++	++	+		2700 on Day 83	
Surgical treatment	1600 mg/day × 5 (8000 mg)	PD	++	+	++	8.9 on Day 20	2100	84,000
Surgical treatment	1800 mg/day × 5 (9000 mg)	PD	+++	+++	+		3000	99,000
Surgical treatment	1600 mg/day × 5 (8000 mg)	PD	+	+	+	9.4 on Day 18	3200	72,000

†PD = progressive disease; NC = no change; Imp = improvement; PR = partial remission (decrease of all measurable lesions by 50% without the appearance of new lesions); CR = complete remission.

‡+ = Mild; ++ = moderate; +++ = severe; ++++ = possible contributing cause of death.

injections were given daily at a starting dose of 350 mg/m²/day for 5 days; this dose level was gradually increased to a maximum tolerated dose of 900 mg/m²/day for 5 days.

Using this new parenteral form, we conducted a clinical trial in which NSC-82196 was given to patients with metastatic malignant melanoma.

MATERIALS AND METHODS

NSC-82196 was given parenterally to 20 patients who had metastatic malignant melanoma. A dose of 900 mg/m²/day × 5 days was given intravenously (iv). The agent was injected slowly in the tubing of a running iv infusion; the total dose administered varied from 7500 to 28,000 mg. All patients entered in the study had confirmed histologic disease with measurable metastases. Prior treatment in 12 patients was surgical only; in an additional eight patients, prior chemotherapy or radiation therapy or both had been given (table 1).

RESULTS

Toxic Effects

Side effects observed included gastrointestinal toxicity, fever, flushing, malaise, local thrombophlebitis, and hematologic toxicity. Flushing was observed in all patients during the administration of the drug. Malaise was noted in 11 patients and thrombophlebitis at the site of injection was seen in all patients.

Gastrointestinal side effects.—Nausea and vomiting were often severe. Two patients had nausea with no vomiting, and a third patient had no nausea or vomiting.

Hematologic side effects.—A fall in the hemoglobin level of more than 2 g/100 ml occurred in six of the 20 patients; this effect was most pronounced within 11–49 days after the treatment had been started. Leukopenia occurred in ten patients; this effect usually occurred about 2 weeks after the start of therapy and persisted for up to 48 days. The lowest white blood cell count was 1500 cells/mm³; it occurred on Day 16 and lasted for 48 days. Thrombocytopenia occurred in ten patients with platelet counts ranging from 99,000 to 41,000 cells/mm³; it lasted from 11 to 21 days. The most severe case of thrombocytopenia (41,000 cells/mm³) occurred after a second course of treatment with NSC-82196. Platelet counts did not fall to less than 60,000 cells/mm³ after the first course of NSC-82196.

Therapeutic Effects

Four of the 20 patients treated showed some response to treatment (table 1). In one patient (No. 9) a complete remission occurred after treatment with NSC-82196. This 48-year-old man had had a primary malignant melanoma which had been removed from the left groin area. He was treated because of extensive skin metastases in the right breast area, as well as right inguinal subcutaneous masses and right axillary lymph node involvement. Within 4 weeks of his first course of 8000 mg of NSC-82196, all measurable lesions had cleared. Except for venous thrombosis at the site of infusion and malaise, no other side effects were observed. A second course of NSC-82196 was then given. After this treatment, the patient's disease again became active and he died 1 month after the start of the second course of treatment.

Partial response with a greater than 50% decrease in measurable lesions occurred in two patients, one who had lymph gland metastases only and one who had lymph gland and skin metastases. In neither of these patients did the response last for more than 2 months. In a fourth patient, improvement lasting 3 months occurred. The response was not good enough to be considered a partial remission.

DISCUSSION

NSC-82196 has shown therapeutic value in the treatment of metastatic malignant melanoma. As judged by the present series of 20 patients, however, therapeutic value is not of a high order. Only one complete remission was noted and responses were of short duration in all four patients who showed any improvement. The toxic effects noted at the dose level used were tolerable but larger doses could not be given because of the possibility of delayed side effects. The agent does not appear to be superior to other cytostatic agents being used at present in the treatment of metastatic malignant melanoma.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Hydroxyurea: NSC-32065

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

NSC-82196: CAS reg. No. 5034-77-5; 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; leurocristine, sulfate (1:1)

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ANNOUNCEMENTS

Proceedings of the Ninth Canadian Cancer Research Conference

The proceedings of the Ninth Canadian Cancer Research Conference (edited by P. G. Scholefield) has been published for the National Cancer Institute of Canada by the University of Toronto Press, 33 East Tupper Street, Buffalo, New York 14203. The price for this 239-page volume is $20.00.

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The principal objective of the program is to increase the number of highly trained physicians and dentists who have special capability in the prevention, diagnosis, therapy, and control of cancer. These Fellowships must be held in institutions with a demonstrated interest and capability throughout all departments to provide for the study and care of cancer patients. Training can be requested for a short-term period or for a period up to 3 years.

Applicants may apply for training in this country or overseas. Only American citizens, persons lawfully admitted for permanent residency at the time of application, or non-citizen nationals may be grantees under this program. Cuban refugees are exempt from U. S. citizenship requirements. Students and persons here on temporary visas are not eligible.

Deadline for application submission is January 2, 1973. Applicants will be notified in June regarding these Fellowships. Stipends are determined on an individual basis.

To apply, write or telephone Mrs. Helen W. Denson, Fellowship Specialist, Division of Cancer Grants, National Cancer Institute, Westwood Building, Room 849, 5333 Westbard Avenue, Bethesda, Maryland 20016 (301-496-7895).

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Children's Hospital of Los Angeles, Department of Pediatrics, University of Southern California School of Medicine

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Candidates interested in fellowship opportunities should write to: Carol B. Hyman, MD, Director, Fellowship Training, or Myron Karon, MD, Head, Division of Hematology, Children's Hospital of Los Angeles, 4650 Sunset Boulevard, Los Angeles, California 90027.

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    - d. "An Outline of Procedures for Preliminary Toxicologic and Pharmacologic Evaluation of Experimental Cancer Chemotherapeutic Agents." April 1964. Compiled by the Cancer Chemotherapy National Service Center.
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